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CONTENTS

PAGE

Carbohydrate Metabolism in the Bovine. I. Intravenous Glucose Tolerance in the Healthy Cow. By J. R. Holmes 1

Carbohydrate Metabolism in the Bovine. II. Intravenous Glucose Tolerance in Various Diseases, including Clinical Ketosis. By J. R. Holmes 15

Studies on Experimental Streptococcal Mastitis. IV. The Disease caused by Inoculation of *Streptococcus agalactiae* Strain S13 into the Teat Canal of Goats. By I. H. Pattison and H. H. Holman... 26

Some Abnormalities of Keratinization in the Skin of Sheep. By Marcia Burns and L. Auber 38

Studies on Respiratory Diseases of Pigs. I. Modified Haemagglutination—Inhibition Technique for Titration of Influenzal Antibodies in Pig and Ferret Sera. By T. S. Gulrajani 48

Studies on Respiratory Diseases of Pigs. II. Antibody Response to Adjuvant Vaccine against Swine Influenza. By T. S. Gulrajani 60

CARBOHYDRATE METABOLISM IN THE BOVINE

I. Intravenous Glucose Tolerance in the Healthy Cow*

By

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INTRODUCTION

The experiments described in this paper were designed to determine the effect of the intravenous administration of glucose to cattle on blood sugar and urinary constituents. They were undertaken during the course of investigations into bovine ketosis which is often associated with a low blood sugar level. One of the aims of this investigation, therefore, was to determine the effect of a given dose of glucose both in normal adult bovines and in cases of clinical ketosis, and in the latter, to assess the effectiveness of this form of therapy. The intravenous route was selected firstly because glucose introduced into the rumen is uncertain in its effects, and secondly because this is a common route of administration in treating clinical ketosis.

It was also decided to investigate the intravenous tolerance test as a method of assessing liver function in bovines. Before the test could be recommended, however, it was necessary to carry out observations on a sufficient number of normal animals of various ages and in various stages of pregnancy and lactation. The results obtained in diseased animals, particularly in cases of suspected liver disease and also in cases of clinical ketosis, will be published in a subsequent paper.

In the human subject, Glucose Tolerance Tests are widely used in the diagnosis of liver disease and diabetes mellitus. In these tests the glucose is usually given by mouth so that the rise occurs primarily in the portal vein and all the absorbed sugar passes through the liver.

The value of the Glucose Tolerance Test in the diagnosis of hepatic disease and the biochemical mechanism involved is well explained by Soskin and Levine (1946). Speaking of the single-stomached animal, they state that "the blood sugar level is a determining factor as regards the activity of the liver in the regulation of carbohydrate metabolism. The characteristic rise and fall of the blood sugar following the administration of dextrose to normal animals, represents a rapid and reproducible test of the regulating mechanisms." The response to the injection of sugar, that is the rate of fall of the induced hyperglycaemia, was originally explained as due to stimulation of insulin production by the injected sugar. Soskin and co-workers refuted this by experiments using (1) completely depancreatised dogs and (2) hepatectomised dogs. These experiments showed that for the normal tolerance curve, the liver was essential but the pancreas was not. Hence they postulated that "in the presence

* This paper formed part of a thesis submitted to the University of Liverpool for the degree of Doctor of Philosophy, May, 1950.

of a sufficiency of insulin, but not necessarily an extra secretion from the pancreas, the normal liver, as one of its responses to administered dextrose, decreases the output of blood sugar which it has previously been supplying from its own resources. This is known as the *Hepatic Homeostatic Mechanism.* The usual period of hypoglycaemia following the induced hyperglycaemia corresponds to the time which elapses before the liver is able to accelerate its rate of supply of blood sugar to a point sufficient to maintain the original level." They state that " the fundamental regulation of the blood sugar is an auto-regulation in which the prime mover is the blood sugar level itself. The liver will go through the same regulating processes at any level of blood sugar depending upon where the homeostatic mechanism is set by the endocrine balance."

The important diagnostic features of the curves are the downslope and the time to return to the pre-injection level. Hence in liver damage such as carcinoma or in toxaemia or diabetes in man, the liver does not respond satisfactorily to the administration of glucose, as a result of which the fall in the sugar curve is much slower than normal.

Intravenous glucose tolerance is occasionally used in man as a diagnostic procedure. The method is described by Soskin and Levine (1946). Administration of dextrose by mouth does not distinguish between hepatic disease and endocrine disturbances such as diabetes mellitus. Using a standard intravenous method Soskin and Levine state that it is possible to obtain curves which are characteristic for each condition and which can be differentiated from each other. In man their test comprises a dose of 1/3 g./Kg. glucose in 20 per cent. solution intravenously on an empty stomach administered within a period of 3 to 5 minutes. Blood samples are collected before administration and at half, one and two hours thereafter. By this method, comparing liver disease with diabetes, it was evident that the downslope of the curve was much more prolonged in diabetes than in liver disease and in the latter more prolonged than in the normal animal. Further, in certain cases it is more desirable to administer the sugar intravenously as for example in suspected hypopituitarism where the rate of absorption from the gastro-intestinal tract is diminished and so may lead to a false response being obtained to glucose administered by mouth. Cantarow and Trumper (1946) noted that in adrenal cortical insufficiency intravenous injection is followed by a sugar rise to an essentially normal level. The hypoglycaemic phase of the curve is, however, generally exaggerated and may remain subnormal for several hours. Hyperinsulinism would produce little or no elevation of blood sugar and exaggeration of the hypoglycaemic phase. They further state that during pregnancy in women there is commonly a decreased glucose tolerance particularly in the later stages. It is seldom extreme and is usually associated with no elevation of the fasting blood sugar level. It is believed to be due to the disturbance of endocrine function (increased thyroid and pituitary activity) which exists during this period.

Once a hyperglycaemia has been induced glycosuria occurs. According to Soskin and Levine (1946) the process by which the kidney tubules reabsorb glucose depends upon the phosphorylating mechanisms. When the amount of glucose filtering through the glomeruli exceeds the phosphorylative capacity of the tubules, glucose appears in the urine. The critical blood sugar level at which this begins to occur is the renal sugar threshold. In these experiments observations could be made on the renal sugar threshold.

METHODS

The Animals used

The animals used were six in number; a Red Poll heifer, a Shorthorn heifer and four Ayrshire cows. All were pregnant and had passed the tuberculin test. A total of 26 tests was carried out.

The Experimental Method

In the early experiments it was necessary not only to determine the frequency with which blood samples should be collected in order to have a satisfactory record of the changes in the blood sugar level in response to the injection, but also to establish a suitable dose of glucose. Bell and Jones (1945) described the effect of administering various doses of glucose to the bovine by mouth, subcutaneously and intravenously, and their results were used as a guide in this matter. It was necessary to provoke a fairly high rise in the blood sugar level in order that the rate of fall could be studied. At the same time samples of urine were obtained and an assessment made of the renal threshold and also the relationship between the amount of glucose injected and the amount lost in the urine. The glucose used was the monohydrate and the concentration in all cases was a 20 per cent. aqueous solution. The dose finally adopted was 0·3 g./Kg. This was found to give very satisfactory results. The animals used were usually weighed before the experiments.

Early experiments indicated that the most satisfactory results could be obtained by collecting blood samples before injection; at two minutes after the *end* of the injection, which was regarded as the maximum rise in blood sugar, and then every 15 minutes for 2½ hours. Occasionally, particularly in the early work, samples were collected at intervals before the test and several hours after the end of the test, but this was found to be unnecessary.

Blood sugar observations have shown that the level in the adult bovine is practically uninfluenced by diet. Consequently no special precautions were taken in these experiments regarding feeding before or during the tests. This statement, however, is not true for calves which resemble more closely the single-stomached animal, as will be shown in a subsequent paper.

The jugular vein was used for the injection and blood sampling. The apparatus used consisted of a litre Pyrex flask with an air inlet tube, and an outlet leading through rubber tubing to an 11 B.W.G. needle. The injection was made by gravity. The procedure consisted in raising the vein with a choke-rope and obtaining a preinjection sample of blood, usually from the left jugular vein. The injection was then given through the large needle into this left vein. With an average volume of 500 to 600 cc. this was completed in 2 to 3 minutes. Samples of blood were thereafter collected from the right jugular in order to prevent contamination from glucose on the skin or in perivascular tissues. For collecting

blood samples, a small-bore needle (17 to 19 gauge) was used. Urine samples were collected by perineal stimulation. The ease with which these samples could be obtained appeared to vary from cow to cow irrespective of the amount of urine present in the bladder. If possible a preinjection sample was collected so that the bladder could be regarded as empty at the time of injection. Thereafter, samples were obtained in a clean bucket at various intervals, generally about every $\frac{1}{2}$ hour, the aim being to collect all urine passed during the period of the test. The volume was measured and samples examined for albumen, ketones, sugar and specific gravity; pH values using Universal Indicator were also obtained. If sugar was present, as shown by Fehling's Test, it was estimated by Benedict's method.

Blood sugar was estimated by the method of Hagedorn-Jensen using Somogyi's modified protein precipitation. The blood samples (about 3 cc.) were collected into clean glass bottles. In order to avoid the need for an anticoagulant and because the laboratory was close at hand, 0·2 ml. was immediately pipetted and laked in 5·8 ml. of distilled water in a test tube. Two samples were always taken from each bleeding for estimation. As soon as possible, and usually within an hour of collection, the proteins were precipitated. The filtrates were then placed in the refrigerator until titrated. Titrations were usually carried out within 24 hours of collection. Observations showed that practically no deterioration occurred provided the blood proteins were removed and the filtrates were kept in the cold.

RESULTS

The intravenous administration of glucose to normal cows

The experiments using glucose in a dose of 0·3 g./Kg. by intravenous injection in normal cows showed that there was generally no hypoglycaemia following the induced hyperglycaemia and that the time taken for the blood sugar to return to the preinjection level varied from $\frac{3}{4}$ hour to $2\frac{1}{2}$ hours; generally occupying $1\frac{1}{2}$ to 2 hours. In practically all cases, the level was normal again within $2\frac{1}{2}$ hours and certainly had returned to within the accepted blood sugar range before that time. In all the experiments, the injection raised the blood sugar above the renal threshold and produced glycosuria. The urine samples contained the greatest concentration of sugar within the first 30 minutes after administration and subsequent samples usually contained progressively decreasing concentrations. The length of time after injection before sugar ceased to appear in the urine was difficult to determine exactly, as it depended largely on the ease and frequency with which samples could be obtained from the cows concerned. In the case of the Red Poll and Shorthorn heifers, from which they were generally easy to obtain, the longest time at

which a sugar positive specimen was recovered was 90 minutes and the minimum period before urine became free of sugar was 60 minutes.

The amount of glucose excreted varied from 2·6 to 20·5 per cent. of the quantity injected; the general range being 6 to 14 per cent. and the average of 22 experiments 10·3 per cent. The

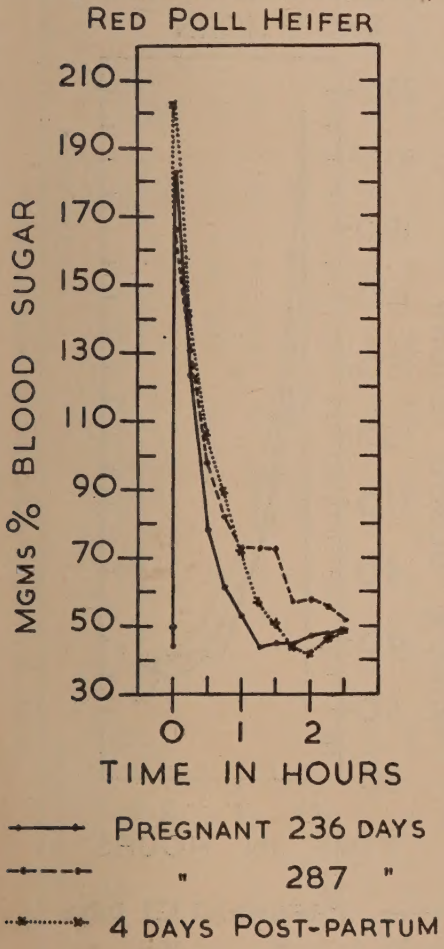


FIG. 1.

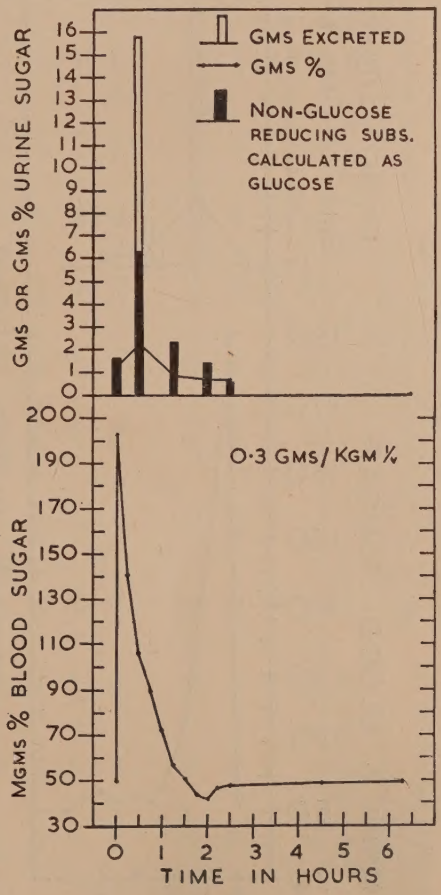


FIG. 2.

FIG. 2. Red Poll Heifer—4 days post partum. All urine samples reduced Fehling's solution and were titrated with Benedict's reagent. It is very unlikely that the reducing substance was glucose in all the samples. Average amount of reducing substance (calculated as glucose) present in preinjection samples and samples 4 and 5 ; 0·86 g. %. Considering this to be the approximate strength of this substance present in samples 2 and 3 and subtracting this from these samples ; then approximate amount of true glucose excreted : 9·5 g. (8·8% of dose).

variation did not appear to be related to the volume of sugar-containing urine excreted, and the differences which occurred in the same cow at different times, while not great, were difficult to understand. This dose of glucose appeared to exert a mild diuretic action for, in general, the urine samples containing the greatest concentration of glucose had the lowest specific gravity. (Tables 1 and 2.)

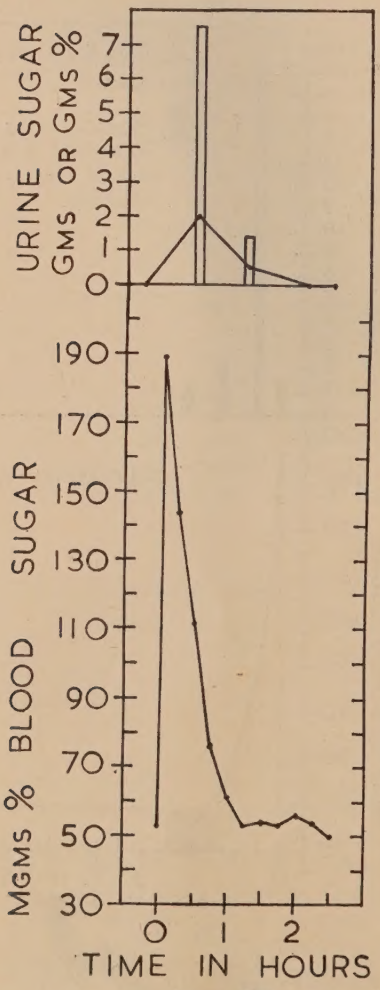


FIG. 3.

FIG. 3. Red Poll Heifer—25 days post partum.

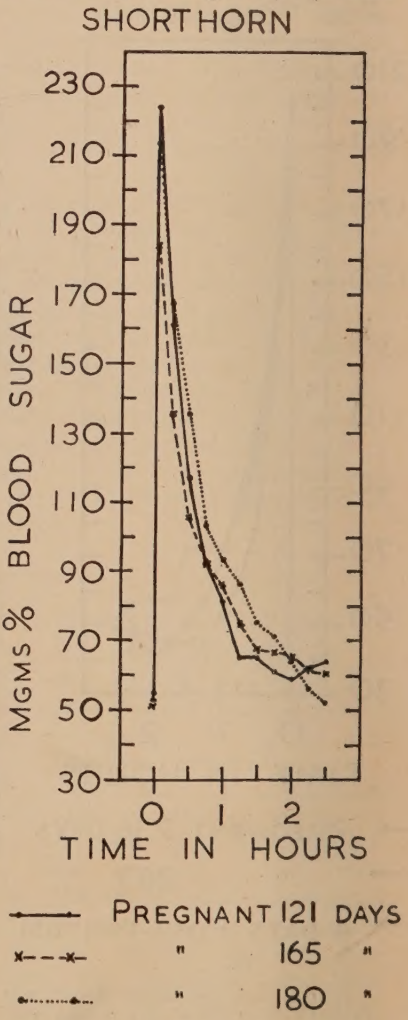


FIG. 4.

The Red Poll heifer showed a reducing substance in its urine in the experiment four days after parturition (Fig. 2). This was

TABLE I

GLUCOSE TOLERANCE TESTS—RED POLL AND SHORTHORN HEIFERS

	BLOOD SUGAR					URINE SUGAR							
Pregnant days.	*Wgt. cwts.*	*Pre-inj level. Mgms. %*	*2 mins. after inj. Mg. %*	*Max. rise Mgms. %*	*Time to return. Hours.*	*Total vol. after injection. (c.c.)*	*During mins.*	*Sugar + ve. urine. (c.c.)*	*During mins.*	*Total sugar excreted. g.*	*Glucose excreted % of dose.*	*S.G.*	*pH.*
RED POLL HEIFER :													
191‡	7	57			¾	890*	150	720*	90	9·2	8·1*	1·034–1·043**	7·0–8·0
213	7½	46			1¼	3850*	150	1,500*	30	5·5	4·8*	1·002–1·015**	7·0–7·5
217	7½	66			¾	2,230	150	890	35	14·2	12·4	1·026–1·041**	5·5–7·0
236	7½	44	183	139	1¼	1,440	145	1,010	65	11·5	10·0	1·023–1·038**	7·0–7·5
283§	7¾	47	193	146	2	1,495	150	1,000	65	8·6	7·3	1·017–1·031	5·0–6·5
287	7¾	50	166¶	116¶	2½	1,555	150	970	50	7·7	6·5	1·017–1·035**	8·0
4 days post partum	7	50	203	153	1½	1,280	150	730‖	30‖	9·5‖	8·8‖	1·024–1·053**	6·5–8·0
25 days post partum	6½	53	189	136	1¼	870	150	640	75	8·9	9·0	1·030–1·043**	8·0
78 days post partum	7	49	211	162	2¼	1,130	155	705	65	10·8	10·1	1·026–1·041**	8·0
89 days†† post partum	6	55	205	150	1¾	440	145	260	65	2·4	2·6	1·011–1·026**	6·0–7·5
SHORTHORN HEIFER :													
121	7½	55	224	169	2	1,025	150	625	30	5·2	4·5	1·006–1·041	5·0–7·5
165	7¾	51	184	133	2½+	810	150	530	50	7·5	6·3	1·029–1·044**	8·0
174†	7¾	42	213	171	2½++	3,470	135	3,210	90	9·4	7·9	1·004–1·017	8·0
180	7½	53	213	160	2½	1,525	145	675	35	4·9	4·2	1·002–1·032	5·0–6·0

Dose in all experiments : 0·3 g./Kg. 20% soln. intrav.

† Immediately after a 48 hour fast.

* Values approximate only.

‡ Fasted when pregnant 183 days to 187 days.

§ Fasted when pregnant 237 days to 239 days and also 269 days to 273 days.

‖ All samples in this experiment were Fehling's + ve. True glucose estimated.

¶ This value is probably a little less than the maximum rise owing to difficulty in collecting the first blood sample.

†† Fasted from 83 to 87 days post partum.

** Min S.G. coincided with the sample containing the greatest concentration of glucose.

TABLE II

GLUCOSE TOLERANCE TESTS—PREGNANT AYRSHIRE COWS

				BLOOD SUGAR				URINE							
Cow No.	*Pregnant days.*	*Dry wks.*	*Wgt. cwts.*	*Pre-injection. Mg. %*	*2 mins. after injection. Mg. %*	*Max. rise. Mg. %*	*Time to return Hours.*	*Total vol. after injection. c.c.*	*During mins.*	*Sugar + ve c.c.*	*During mins.*	*Total sugar excreted g.*	*Glucose excreted % of dose*	*S.G.*	*pH*
79	239	2	11	59	174	115	2½+	2,650	110	2,650	110	21·9	13·0	1·033–1·036	8·0
79	277	7	11¼	63	181	118	1¾	1,935	125	1,000	35	13·0	7·5	1·021–1·037§	7·5–8·0
87	255	7	9	52	174	122	2	1,320	135	1,285	65	14·9	10·8	1·033	8·0
87	274	10	9¾	57	168	111	2	2,595	140	2,595	140	19·9	13·3	1·021–1·028	8·0
105	164	3	10½	53	197	144	1	3,135*	145	2,305*	50	16·5*	10·2*	1·011–1·038	8·0
105	193	8	11½	49	204	155	1¾	3,275	70	2,985	50	36·0	20·5	1·007–1·023§	8·0
105	224	12	11¾	46	201	155	2¼	2,395	140	1,980	65	26·4	14·7	1·022–1·038	8·0
105	253	16	12½	52	181	129	1¾	3,470	95	2,270	35	26·5	13·8	1·023–1·047§	8·0
105	274	19	12½	53	161	128	1	3,195	140	3,090	65	21·1	11·0	1·016–1·034	8·0
105	189†	7	11½	50	142	92	1+	9,515	110	4,190	30	20·8	14·0	1·014–1·024	8·0
108	251	3	9	77‡	171	94	1¼	4,250	150	3,500	55	23·5	17·1	1·003–1·027	5·0–8·0
108	278	7	10¼	50	142	92	2½	1,090	125	980	75	15·9	10·1	1·031–1·043§	8·0

* Volume approx. only.

† All doses 0·3 g./Kg. 20% soln. intrav. except in this exp. when the dose was 0·25 g/Kg.

‡ Cow difficult to restrain so level probably higher than normal.

§ Min S.G. in sample containing the greatest concentration of glucose.

not glucose and it is suggested that reabsorbed lactose from the mammary gland may have been involved. Certainly parturition or the onset of lactation appeared to be related to its appearance.

Glucose tolerance with advancing pregnancy

In an investigation of the effect of advancing pregnancy on glucose tolerance, four Ayrshire cows were used. Three of these cows were tested twice and one cow had six tests. (Figs. 5-8 and Table II.) These experiments were also carried out for a further reason. The cows were carrying their third or fourth calves and all were due to calve in the winter or early spring. Experience had shown that they would be more liable to develop ketosis when calving during this period. If, therefore, ketosis is associated with changes in liver function which develop during advancing pregnancy, then possibly these changes would result in alterations in glucose tolerance which could be related to any metabolic upset which might develop after parturition. The past histories of all

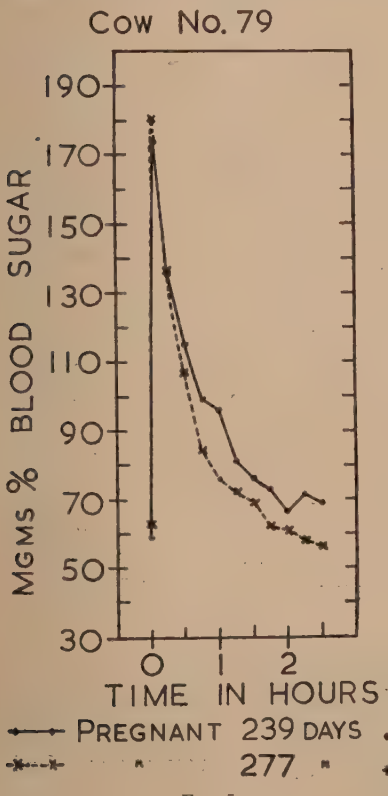


Fig. 5.

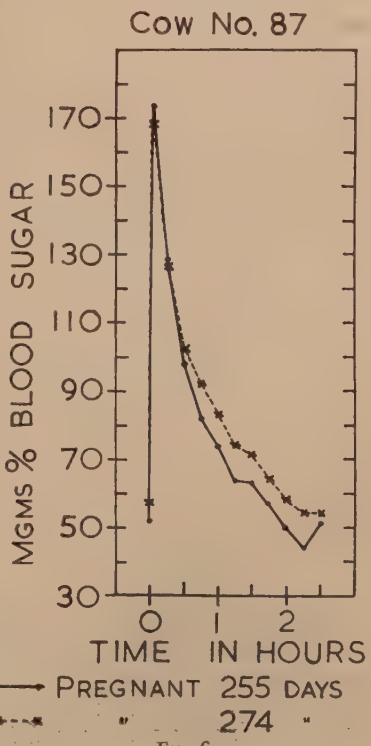
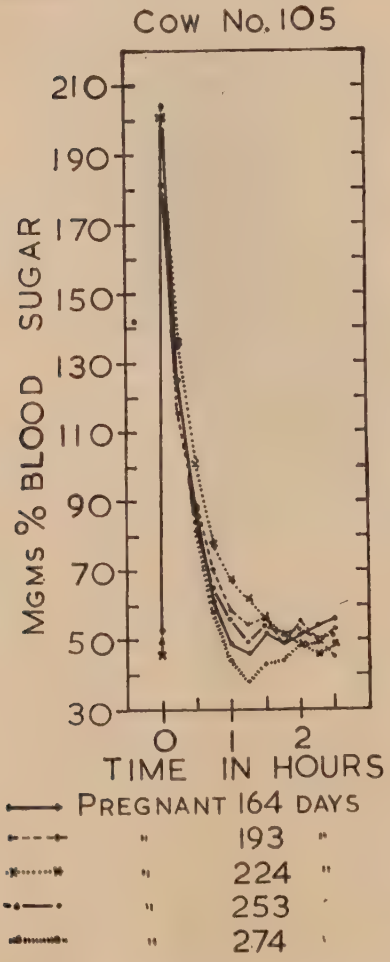


Fig. 6.

these animals in respect of disease, milk yield and dry periods were available, and of the four cows chosen, one animal had previously suffered from ketosis (Table III). These experiments did not indicate any marked change in glucose tolerance with approaching term.



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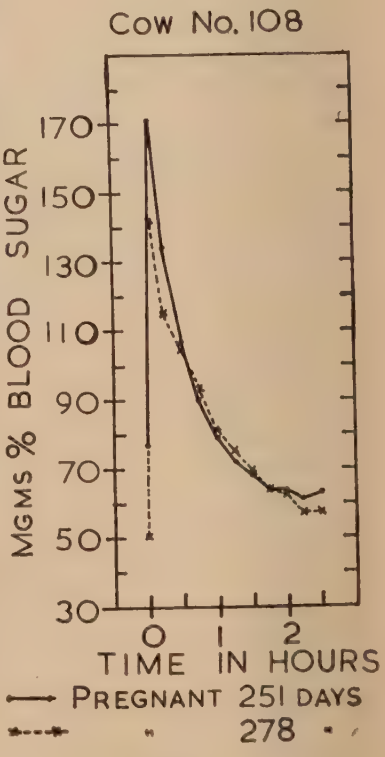


FIG. 8.

It is interesting to note that cow No. 79 (Fig. 5) showed a slower fall on first test when pregnant 239 days than on second test when pregnant 277 days when the blood glucose fell more rapidly. The writer suggests that this may be related to the fact that this cow had just completed a very heavy lactation and had only been dry two weeks when first tested.

TABLE III

EXPERIMENTS TO DETERMINE THE EFFECT OF ADVANCING PREGNANCY ON GLUCOSE TOLERANCE.

History of the four cows used.

Cow No.	*Calved.*	*Lactation.* (*lbs.*)	*Dry period.* (*wks.*)
79	1.3.45	7288	28
79	26.6.46	10401	28
79	11.1.48	10198	Dry
87	Nov. 1945	8847	12
87	20.12.46	7258	17
87	29.11.47	7616	Dry
105	18.12.46	7399	11
105	16.1.48	7353	Dry
108	3.1.47	8453	7
108	9.1.48	7978	Dry

Of these animals No. 87 had previously suffered from ketosis in December, 1947.

History after testing.

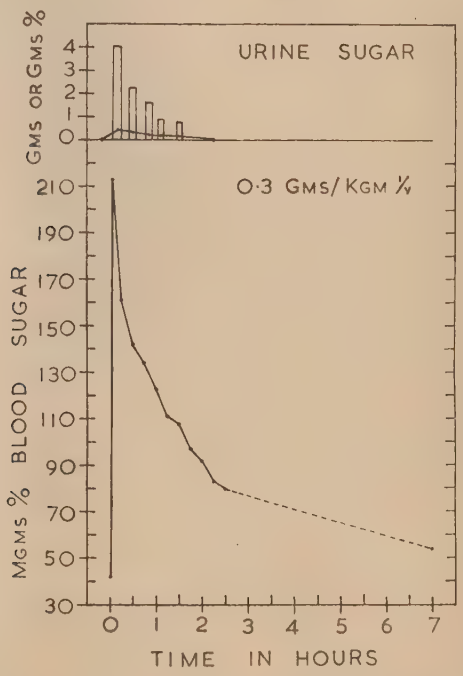
Cow No.	*Calved*	*Metabolic Disease.*
79	23.12.48	None.
87	13.12.48	Milk Fever 6½ hours after parturition. Responded to 3-ozs. Ca. boroglu-conate.
105	6.3.49	None
108	22.12.48	None

Glucose tolerance in pregnancy plus fasting

A Red Poll heifer was used in the early part of the work and this animal was also utilized for experiments on the effect of fasting during pregnancy. Whilst no glucose tolerance tests were carried out earlier than two days after the end of a fast, glucose tolerance appeared to decrease as pregnancy advanced (Fig. 1). It

was evident that this might have been due to pregnancy or to fasting or to both.

A striking change in response followed parturition. At first sight, it appeared that glucose tolerance in the bovine decreases with advancing pregnancy but during this period this heifer had been subjected to fasting for two periods of four days and one period of two days. In considering the effect of fasting on glucose tolerance a Shorthorn heifer was fasted for two days and this was ended by an intravenous injection of glucose (Fig. 9). The results



EFFECT OF FASTING ON GLUCOSE TOLERANCE

FIG. 9.

Shorthorn Heifer; pregnant 174 days. Injected immediately after a 48 hour fast.

indicated a definite decreased tolerance for sugar, the rate of fall of the blood sugar level being slower than normal. As the experiments on the Ayrshire cows showed that pregnancy did not appreciably alter tolerance for glucose, it is considered that the decreased tolerance shown by the Red Poll heifer as pregnancy advanced was associated with fasting during pregnancy and not with pregnancy *per se*.

DISCUSSION

The results indicate that the intravenous injection of glucose monohydrate to the adult bovine in a dose of 0·3 g./Kg. in a 20 per cent. aqueous solution results in an elevation of the blood sugar level with glycosuria. The induced hyperglycaemia quickly falls and is not followed by a period of hypoglycaemia. Pregnancy does not appear to be associated with any marked change in glucose tolerance but it would appear that fasting during pregnancy results in a decreased tolerance for glucose.

An interesting feature of the tests on the four Ayrshire cows is the "individual" nature of the response in each animal. The four cows are really each quite different from one another in their response to the same dose of glucose. The individual animals appear to respond in a similar manner each time to the administered sugar. Each cow appears to have a blood sugar rise and rate of fall which are more or less characteristic of itself and its own metabolic processes. This illustrates the danger of generalisations in the metabolic problems of domestic animals. The cow must be considered as an individual creature as well as a member of a herd community. The writer, however, admits that this suggestion is still rather controversial and shows the difficulty of separating the general from the particular in analysing these results. The problem of bovine glucose tolerance requires further investigation, possibly using castrated males free from the metabolic stress of pregnancy and lactation in order to establish a "base line" or characteristic response for what may be regarded as a "metabolically neutral" animal, before these results can be properly interpreted. Cow No. 87 developed Milk Fever after calving, but the glucose tolerance tests taken before parturition (last test five days before calving) did not vary from normal. None of the other animals showed any metabolic disturbance, so that it is not possible to determine whether, if ketosis had developed, the pre-parturition glucose tolerance tests would have shown any variations from normal.

Observations were not made specifically to determine the renal sugar threshold in the adult animal, so that an approximate range only can be suggested. It would appear to lie in the region of 90 to 100 mg. per cent., as is shown by the experiment on the Shorthorn heifer (Fig. 9). There is no doubt that the renal threshold varies slightly from animal to animal and the threshold in the calf must be much higher than in the adult. As the rumen develops and the animal becomes a true herbivore the blood sugar level slowly falls and the renal threshold must also be reduced.

Bell and Jones (1945) found the ascending renal threshold in adult cattle to be 98 mg. per cent. and the descending mean was 102 mg. per cent. These observations would support their findings.

SUMMARY

Observations are recorded on the intravenous glucose tolerance of the healthy cow. Six animals were used in the investigation and a total of 26 tests was carried out. A standard dose of 0·3 g./Kg. in 20 per cent. aqueous solution was used. The relationship between the amount injected and the glucose lost in the urine was obtained. The possible value of the method as a liver function test in the bovine is considered.

ACKNOWLEDGMENTS

I wish to express my thanks to Professors R. A. Morton and J. G. Wright for their help and interest in this work, which was carried out whilst the writer was the holder of the A. D. Allen Memorial Scholarship of the Animal Health Trust. The work forms part of the results of a two-year programme of research into Bovine Ketosis which was aided by grants from the Agricultural Research Council and the Animal Health Trust.

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CARBOHYDRATE METABOLISM IN THE BOVINE:

II. Intravenous Glucose Tolerance in Various Diseases, including Clinical Ketosis*

By

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INTRODUCTION

Intravenous glucose tolerance in normal cows has been considered in a previous paper (Holmes, 1951). The possible value of the method as a hepatic function test and the mechanisms involved was then discussed. In this paper the results of administering glucose intravenously to diseased animals is considered. In regard to the cases of clinical ketosis this test was applied for three reasons: first, in order to determine if an abnormal tolerance would result, which might indicate that clinical ketosis is associated with impairment of liver function; secondly, to determine whether, following the induced hyperglycaemia the blood sugar level will remain in the normal range or return to the previous hypoglycaemic level; and thirdly, to investigate the duration of the effect of glucose administered intravenously in order to assess the value of this form of therapy. The results indicated that the effect of an intravenous injection lasted 2 to $2\frac{1}{2}$ hours only, the blood sugar then returning to the pre-injection hypoglycaemic value. It was therefore decided in one case to give two injections of glucose with a two-hour interval between and in one experiment glucose was injected three times in six hours.

Soskin and Levine (1946) state that "when dextrose tolerance tests are repeated in a normal animal, each test starting as soon as the previous one is over, the second curve is lower (or better) than the first and the third better than the second. Subsequent improvement usually does not occur. The lowering of the second and third curves in the normal animal can best be explained as a progressive depression in the activity of the pituitary gland as a result of repeated or prolonged exposure to hyperglycaemia. This phenomenon does not occur in the hypophysectomized animal, in which the first curve is the lowest".

It was thought that observations on the triple glucose tolerance response in clinical ketosis might indicate whether pituitary dysfunction was a factor in the etiology of this condition.

METHODS

The Animals used in these Experiments

The animals were sent to the hospital of the Veterinary Field

* This paper forms part of a thesis on Bovine Ketosis submitted to the University of Liverpool for the degree of Ph.D.

Station of the University. In the non-ketosis group three animals were used comprising cases of unthriftiness and suspected hepatic dysfunction, traumatic pericarditis, and suspected Johne's Disease respectively. In the ketosis group there were also three animals.

The Experimental Method

The injections were made and blood and urine samples collected and examined as described previously (Holmes, 1951). The dose of glucose used was 0·3 g./Kg. in 20 per cent. aqueous solution. Unfortunately it was not possible to weigh the hospital cases so their weights had to be estimated in computing the required dose. Milk was examined using the semi-quantitative method described by Patterson (1945) using a mixture of solid ammonium sulphate and sodium nitroprusside. The acetone value of serum was estimated approximately, employing a mixture of ammonium sulphate, sodium nitroprusside and anhydrous sodium carbonate, and using a spot method.

OBSERVATIONS

Non-ketosis cases

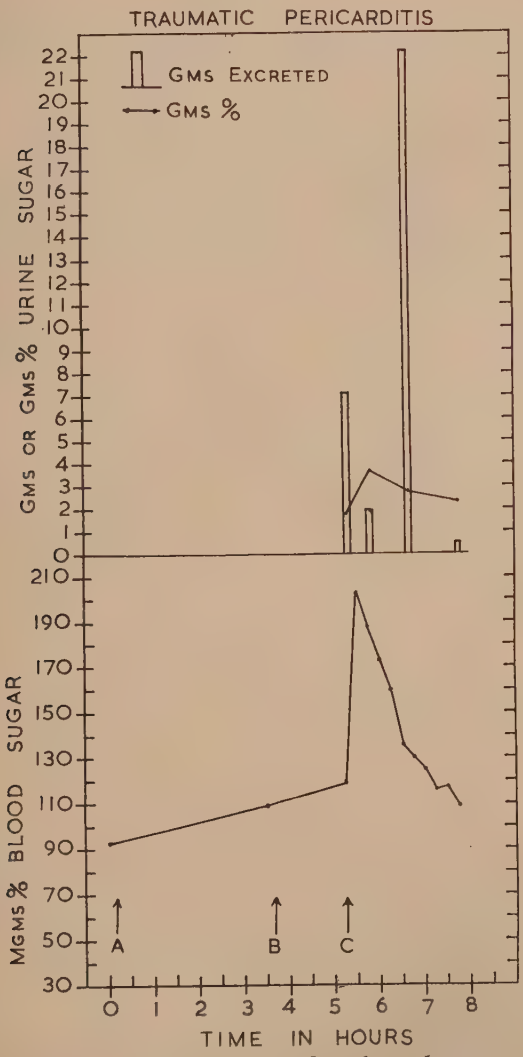
No. 662.

A non-pregnant Welsh Black heifer weighing about 7 cwt., 2½ years of age, suffering from traumatic pericarditis.

A blood sample taken immediately before operation showed a blood sugar level of 93 mg./100 ml. (at A on Fig. 1). Two operations were performed; the first a rumenotomy under local anaesthesia when a piece of wire was removed from the reticulum and the second five hours later under intravenous chloral hydrate narcosis and local infiltration when about 1 gallon of pus was eradicated from the pericardial sac. Immediately before the intravenous injection of chloral hydrate for the second operation, the blood sugar was 109 mg./100 ml. (at B, Fig. 7). One and three-quarter hours later the blood sugar was 119 mg./100 ml. Glucose solution (0·3 g./Kg.) was then injected intravenously. The time required for a return to the pre-injection level was two hours, a figure of 109 mg. per cent. being recorded at 2½ hours. During this latter period four samples of urine with a total volume of 1,280 cc. were collected. Glucose excreted was 31·7 g. i.e. 27·8 per cent. of the dose. Although some of the reducing substance may have been due to the chloral most was no doubt glucose. All samples were positive for albumen and negative for ketones. The S.G. of the samples was 1·027 and the pH was 6·0 to 7·5. Four and a half days later a second injection of 0·3 g./kg. glucose was given (Fig. 2). The preinjection blood sugar level at this stage was 64 mg./100 ml. and the time taken to return to this value was one hour. Three samples of urine were collected in the three hours after injection. The total volume was 960 cc. of which 920 cc. contained glucose. Total glucose excreted was 13·3 g. which was 11·6 per cent. of the dose. All samples were positive for albumen

and negative for ketones. The S.G. was 1·017 to 1·052 and the pH 6·0 to 6·5. The minimum S.G. coincided with the sample containing the greatest concentration of sugar.

Fig. 1



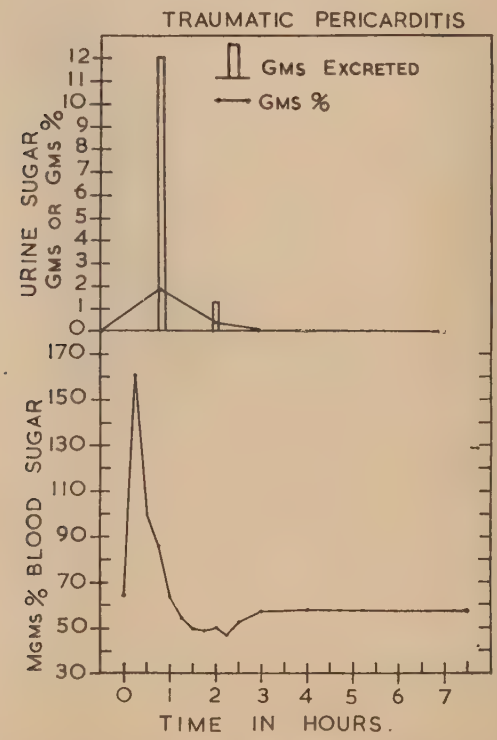
The animal was destroyed two days later because it failed to improve. *Post-mortem* examination showed gross pericarditis and a spreading pleurisy. Histologically the liver showed a monolobular cirrhosis with new bile duct formation. In parts, the con-

nective tissue was pericellular. The liver cells were undergoing degeneration. A few areas of round celled infiltration were present.

No. 670.

An unthrifty, non-pregnant Guernsey cow showed marked diarrhoea, Johne's disease being suspected. The estimated weight was 8 cwt. The animal was given 0·3 g./kg. glucose intravenously.

Fig. 2



The pre-injection blood sugar level was 45 mg./100 ml. and the level returned to this value 1¼ hours after injection in an almost identical manner with the previous case. 1,600 cc. of urine in four specimens was obtained in 2½ hours of which 1,530 cc. contained glucose. The glucose excreted was 13·2 g. i.e. 10·8 per cent. of the dose. All samples were negative for albumen and ketones. The S.G. was 1·031 to 1·040 and the pH 7·0 to 8·0.

No. 690.

This was an unthrifty, non-pregnant lactating Ayrshire cow, whose weight was estimated as 8 cwt. She was in poor condition, but there was no evidence of tuberculosis, Johne's disease or para-

sitic infestation. Hepatic dysfunction was considered as a possible cause. During the 27 days the animal was under observation body temperature range was 100·8 to 103° F. The pulse rates varied from 59 to 84 with an average of 70.

First Test. Following the intravenous injection of glucose the blood sugar returned to the pre-injection level of 57 mg./100 ml. in 2 hours. Three urine samples were collected during 2½ hours after dosing, comprising a total of 2,130 cc. All samples were positive to Fehling's Test and negative for albumen and ketones. The S.G. varied from 1·014 to 1·047 and the pH was 6·5 to 7·5.

Second Test. After a similar dose of glucose had been injected, the blood sugar level had returned to the pre-injection value of 53 mg./100 ml. in 2½ hours. Again three urine samples were collected during the 2½ hours, the total volume being 910 cc. and all were positive to Fehling's Test and negative for albumen and ketones. The S.G. range was 1·020 to 1·048 and pH 7·0 to 8·0.

All urine samples from this animal, even when obtained at times remote from the glucose injections and when the blood sugar was normal, contained a substance which would reduce Fehling's solution. The amount present, calculated as glucose after titration with Benedict's reagent, varied from 0·6 to 2·8 g. per cent. (10 samples). The nature of this substance was not determined. An osazone could not be formed. Hence it is only possible to estimate the approximate amount of glucose excreted. In the first test, 45 minutes after injection the blood sugar was 91 mg. per cent.; 10·9 g. reducing substance calculated as glucose had been excreted which represented 8·9 per cent. of the dose. In the second test at the same time after injection the blood sugar was 90 mg. per cent.: 9·9 g. reducing substance calculated as glucose was excreted representing 8·1 per cent. of the dose.

Of the three animals studied, one (No. 662) showed a decreased glucose tolerance on first test, while on second test the response was normal. The other two gave results similar to those obtained in healthy cows.

Ketosis Cases

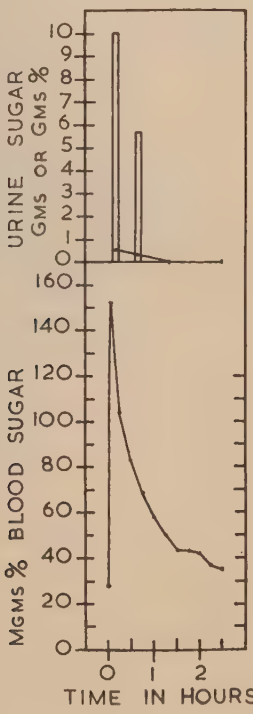
Ayrshire cow R.3.

This cow which was in poor condition and had an estimated weight of 8 cwt., calved her third calf on 10th January, 1949, and developed symptoms of ketosis on 28th January, 1949. It was reported that she had ketosis after her previous parturition. There was a strong smell of acetone in the breath; the faeces were firm in consistency but the cow was not constipated. Milk yield had fallen from 43 to 25 lbs. daily.

The cow had been given ½ lb. of glucose subcutaneously by the attending veterinary surgeon on 29th January, 1949, 31st January, 1949, and 3rd February, 1949, and had been turned out to pasture during the daytime. No appreciable improvement resulted from this treatment. On 5th February, 1949, the cow was given 0·3 g./Kg. glucose in 20 per cent. solution intravenously (Fig. 3). The

pre-injection blood sugar level was 28 mg./100 ml. and $2\frac{1}{2}$ hours later it was 35 mg./100 ml. Four urine samples (6,060 cc.) were obtained during the $2\frac{1}{2}$ hours, of which 3,885 cc. contained glucose. The total glucose excreted was 15·7 g. which represented 11·0 per cent. of the dose. All the samples were negative for albumen and positive for ketones. The first three samples were Rothera positive to a 1/80 dilution and the last sample went to 1/200. The S.G. range was 1·007 to 1·017 and the pH was approximately 5·0. The milk contained approximately 20 mg. per cent. acetone and the serum 50 mg. per cent.

Fig. 3



Fresian cow 411 J.

This cow, which had suffered from ketosis in March, 1949, weighed about 11 cwt. She was in good condition and calved her fourth calf on 15th March, 1949. Ketosis developed on 23rd March, 1949. There was a strong smell of acetone in the breath. Appetite for concentrates was irregular but she was eating hay and occasionally mangolds and silage. Milk yield had fallen from 41 to 27 lb. daily. On 28th March, 1949, 4 oz. of glucose had been given intravenously and on the following day 0·3 g./Kg. glucose in a 20 per cent. solution. The pre-injection blood sugar level was 28 mg./100

ml. and 2½ hours after dosing the level was 35 mg./100 ml. which was almost identical with the previous case. Only one urine sample comprising 1,830 cc. could be obtained during the 2½ hours. It contained 10·6 g. of glucose or 6·2 per cent. of the dose. The urine was negative for albumen and positive for acetone to a dilution of 1/400. The S.G. was 1·038 and the pH 7·5. The milk contained approximately 15 mg. per cent. acetone and the serum approximately 35 mg. per cent.

Shorthorn cow 758.

This cow also had ketosis in March, 1948. She calved her fourth calf on 6th February, 1949, and symptoms of ketosis appeared on 13th February, 1949. The animal was in fair condition with an estimated weight of 9 cwt. Concentrates were completely refused but a little hay was eaten. There was a marked smell of acetone in the breath. Faeces were rather soft for a typical ketosis case. The milk yield had fallen from 55 to 26 lbs. daily.

Single glucose tolerance test. Glucose in 20 per cent. solution (0·3 g./Kg.) was given intravenously on 17th February, 1949. The pre-injection blood sugar was 40 mg./100 ml. and 2½ hours after injection it was 49 mg./100 ml. Five urine samples of a total volume of 2,650 cc. were collected during seven hours after dosing. All the urine passed during the first 2½ hours (1,140 cc.) contained glucose. The total glucose excreted was 10·9 g. or 7·6 per cent. of the dose. All samples were negative for albumen and positive for ketones the range for the latter being 1/450 to 1/200 dilution. The S.G. range was 1·024 to 1·036 and pH varied from 6·5 to 7·0. The milk contained approximately 15 mg. per cent. acetone and the serum 25 mg./100 ml. No clinical improvement was evident following this treatment.

Double glucose tolerance test. Two intravenous injections of glucose were given on the following day with a two-hour interval between. The response may be tabulated thus: —

Blood Sugar.	*1st injection.*	*2nd injection.*
Pre-injection level ...	45	51
Maximum rise	131	125

The level at 2½ hours after the second injection was 49 mg./100 ml. Five urine samples were collected during 7 hours following the first injection, the total volume being 4,880 cc. of which 1,715 cc. contained glucose. The glucose excreted after the first injection was 10·7 g. (7·5 per cent. of dose), and after the second was 7·5 g. (5·2 per cent. of dose). All samples were negative for albumen and

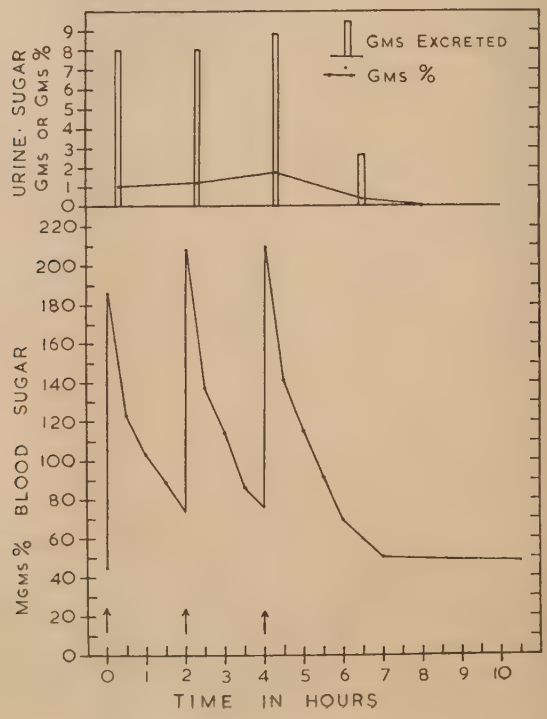
positive for ketones the range for the latter being 1/400 to 1/100 dilution. The S.G. varied from 1·009 to 1·039 and the pH from 6·0 to 8·0. The milk contained approximately 15 mg. per cent. acetone and the serum 15 mg. per cent. On 19th February, 1949, the cow commenced to eat concentrates and was also eating kale and hay. There was a slight smell of acetone in the breath.

Triple glucose tolerance test. On the 21st February, 1949, the cow was given three intravenous injections of glucose with a two hour interval between each.

Blood Sugar.	*1st Injection.*	*2nd Injection.*	*3rd Injection.*
Pre-injection level ...	45	74	71
Maximum rise	141	134	133

The level three hours after the third injection had fallen to 50 mg./100 ml. Five urine samples were obtained, the total volume being 3,215 cc. of which 2,680 cc. contained glucose. The

FIG. 4



glucose excreted after the first injection was 8·0 g. (5·6 per cent. of dose); after the second injection was 8·0 g. (5·6 per cent. of dose); and after the third injection was 8·8 g. (6·1 per cent. of dose) (Fig. 4). All samples were negative for albumen. The first three samples were acetone positive to a dilution of 1/50 to 1/5. The S.G. was 1·014 to 1·030 and the pH 5·5 to 7·0. The milk contained approximately 5 mg. per cent. acetone and the serum 10 mg. per cent.

All the responses showed no striking difference from those observed in normal animals. Two of the cases (R3 and 411J) had marked hypoglycaemia at the time of examination.

Non-Ketosis Cases

DISCUSSION

It was unfortunate that of the three cases examined liver material was available from one animal only. Nevertheless, certain observations may be made.

The results obtained in the Welsh Black heifer (Case No. 662) are interesting and worthy of comment. The animal immediately after the pericardotomy operation showed a marked decrease in tolerance. This would be expected in a case of gross toxaemia with a consequent interference with liver function. Two important facts must, however, be considered. In the first place the initial blood sugar was high and hyperglycaemia was present following two operations. In the second the animal was given 25 g. chloral hydrate intravenously before the pericardotomy operation. It is quite possible that apart from the toxaemia, two factors were acting together to produce a very slow fall in the induced hyperglycaemia, namely the production of adrenaline consequent upon casting and interference and the effect of the chloral tending to produce a higher level of reducing substances in the blood and hence a false high blood sugar level. Further, chloral detoxication and excretion would be delayed by toxaemia.

The animal gave a normal response to injected glucose $4\frac{1}{2}$ days after the operation when it may be supposed to have obtained relief from the toxaemia. It died subsequently and on *post mortem*, there was a very definite cirrhosis of the liver. Hence, while it may be that the delayed fall in the first test was due to interference with liver function, the other possibilities must be considered and a definite conclusion as to the exact factor or factors concerned cannot be drawn.

The case of suspected Johne's Disease gave a normal response to injected sugar as did the unthrifty cow, but the latter animal showed a reducing substance in her urine. It is unlikely that this was glucose because no osazone could be produced; moreover, the blood sugar level was within the normal range and samples containing this substance in greatest concentration usually had a high S.G. The opposite was the case in glucose-containing urine. The nature of the substance was not determined. It is possible that reabsorbed lactose from the mammary gland was involved.

The value of this method as a liver function test cannot be

assessed from these few experiments and further observations are required before definite conclusions may be formed as to its significance. As the liver has considerable reserve tissue and powers of regeneration it is likely that an abnormal response to glucose injection would only be evident in cases of gross liver damage.

Ketosis cases

The glucose tolerance of the three cases investigated was normal. This indicates that the animals can metabolise glucose irrespective of the high level of blood ketones, for the proportion of the dose which was excreted was similar to that observed in normal animals. The double and triple glucose tolerance tests did not indicate any endocrine dysfunction. Cases R3 and 411J showed marked hypoglycaemia and in them the blood sugar returned to the hypoglycaemic level after injection. Although case No. 758 did not show hypoglycaemia, the blood sugar level was in the lower ranges of normal and the normal value (approximately 50 mg./100 ml.) was not maintained even after repeated intravenous injections. The observations indicate the very transitory effect of glucose when administered intravenously. Following the injection there is usually a temporary fall in the urinary concentration of ketone bodies and in the serum acetone level, but this may be partially due to the temporary increase in blood and urinary volume.

The fact that the animals tested gave a response similar to that observed in normal animals does not, of course, rule out the possibility of liver dysfunction being a very important factor in the development of clinical ketosis. The writer is inclined to the view that the symptoms of ketosis are largely due to changes within the liver consequent upon a disturbance of the glycogen-fat ratio. If this is true then treatment should aim to restore normal liver function.

Soskin and Levine (1946) state "the blood sugar concentration and not the amount of glucose administered must be regarded as important for the rate of glycogen deposition in the liver. Hence to protect a damaged liver, the blood sugar concentration may have to be raised to a level possible only by intravenous injection. The fact that extreme hyperglycaemia so produced may lead to glycosuria should not deter one from such vigorous therapy". Shaw and Ross (1941) found that "the intravenous injection of glucose in most cases of severe ketosis usually produces a small decrease in blood and urinary acetone-bodies for a few hours, followed by a rise often to the previous level", whilst Shaw, Powell and White (1942) noted that to promote a rapid and permanent recovery with glucose it must be administered at frequent intervals and in substantial quantities over a period of time.

It is possible that the subcutaneous method of administering glucose may be more useful than the intravenous route owing to its extended reaction.

SUMMARY

The intravenous glucose tolerance of three diseased animals is recorded. The observations were made in an attempt to assess the value of the method as a liver function test. Three ketosis cases are also described in which glucose was given intravenously. No marked change from normal in glucose tolerance was observed. The effect on the blood sugar level was transient and it is suggested that therapeutically subcutaneous administration may be of more value owing to slower and hence more prolonged absorption.

ACKNOWLEDGMENTS

My thanks are due to Professors R. A. Morton and J. G. Wright for their help and encouragement throughout this work and also to Mr. W. Kendrick, M.R.C.V.S., of Tarvin, for supplying the clinical cases of ketosis. The report on the histology of the liver (case 662), was kindly supplied by the Department of Veterinary Pathology. This work forms part of a two-year programme of research into Bovine Ketosis which was aided by grants from the Agricultural Research Council and the Animal Health Trust during which the writer was the holder of the A. D. Allen Memorial Scholarship of the Animal Health Trust.

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STUDIES ON EXPERIMENTAL STREPTOCOCCAL MASTITIS

IV. The Disease caused by Inoculation of *Streptococcus Agalactiae* Strain S 13 into the Teat Canal of Goats

By

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INTRODUCTION

Pattison, Taylor and Holman (1950) and Taylor, Holman and Pattison (1950) described the effects, over a period of nine days, of inoculation of *Str. agalactiae* strain S 13 into the teat canal of goats. It was concluded that the inoculated animals were affected with a disease that was clinically, bacteriologically and biochemically similar to bovine streptococcal mastitis.

The purpose of the present paper is to elaborate on the observations made in the papers referred to above, to extend the description of the disease up to a period of eleven weeks after inoculation and to present and summarise all available data on the disease produced by intramammary inoculation of goats with *Str. agalactiae*. The underlying object of this rather detailed description of an artificial disease is to make the information available as a foundation for chemotherapeutic, immunological and pathological studies.

MATERIALS AND METHODS

The general methods employed were described in the papers referred to above. Cross-bred goats of various ages, all in full lactation and all housed indoors, were used for the various experiments. All were inoculated into one side of the udder only, and all were destroyed for histopathological examination at the end of the experimental period. The haematological, bacteriological and biochemical methods used have been described previously. In making Breed smears for counting leucocytes, 0·01 ml. of mixed fresh milk was spread from a wire loop over 1 sq. cm. of a glass slide; the number of cells present in 0·25 sq. mm. was counted (21 fields under a 1/12th objective) and from this figure the number of cells per ml. of milk was calculated. Classification and enumeration of degenerate cells in milk smears can be a complex procedure (Zlotnik, 1947), and for this reason only cells that appeared to be healthy neutrophils were counted. There was a rise in cell count in the milk from the inoculated side of all udders, but in some goats post-inoculation counts did not exceed pre-inoculation counts for other goats. If a count of one million cells per ml. was taken as the level of normality, all pre-inoculation counts and 17 per cent. of post-inoculation counts were below this figure.

Technique of Infection

In previous experiments, the doses of living organisms inoculated varied from about 1,000 to about 100,000 million, each being made up to a volume of 10 ml. by addition of supernatant fluid. This supernatant fluid, obtained by centrifugalization, was water clear but was known to contain streptococci. All these inoculations were followed within eight hours by a sharp systematic reaction. On one occasion (see Taylor *at al.*) the inoculation of a faulty filtrate containing what was presumed to be a very small number of living organisms was followed by a delayed reaction.

In view of these findings, it seemed possible that the inoculation of a small number of living organisms might always be followed by a delayed reaction, and that by adding some sterile medium, *e.g.* broth, instead of supernatant fluid to the basic dilution containing 1,000 organisms, an inoculum with this action could be obtained. It was thought that such an inoculum would simulate more closely natural field infection with *Str. agalactiae.*

The series of experiments carried out to examine the speed and type of reaction to various kinds of inocula does not warrant detailed description, but it was found that inocula made up with supernatant fluid, or containing large numbers of organisms washed in broth, acted rapidly and produced clinical mastitis in all 25 goats inoculated. By contrast, inocula of about 1,000 living organisms made up to 10 ml. with some sterile medium always produced a delayed reaction and infected 25 out of 26 goats inoculated. A dose of about 1,000 living streptococci was conveniently obtained by using 3·0 ml. of a 10^{-6} dilution in saline of an 18-hour Todd-Hewitt broth culture.

It seemed, therefore, that the rapidity of the reaction of goats to inoculation depended on the number of living organisms inoculated. The delayed type of reaction presumably resembled most closely natural field infection with *Str. agalactiae,* and for this reason the standard inoculum used for long-term study of the disease in goats was of about 1,000 living organisms in 3·0 ml. of saline made up to 10 ml. with Todd-Hewitt broth.

RESULTS

The Course of the Disease over Eleven Weeks

Ten animals inoculated into one half of the udder with the delayed action inoculum and two animals similarly inoculated with heat-killed organisms were kept under observation for periods varying up to 11 weeks after inoculation.

The only abnormality detected in the uninoculated side of any udder was a small, transient drop in yield in some cases. Other reactions were as follows: —

Reaction to heat-killed organisms

One of the two animals inoculated with heat-killed organisms showed a rise in temperature to 104·3° F. on the fifth day, with

some swelling of the inoculated side of the udder at that time and a rise in milk leucocyte count. Figure 1 shows the results for this animal over the eleven-week period.

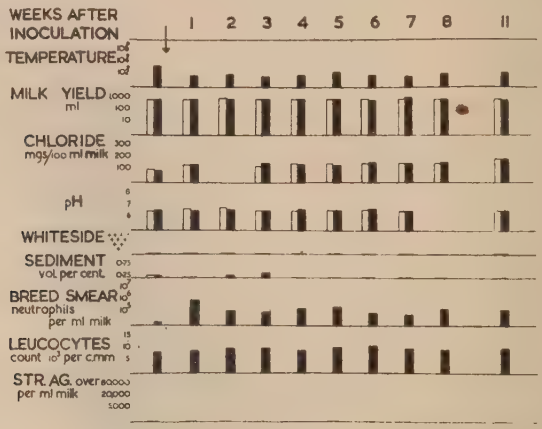


FIG. 1. Typical reaction to intramammary inoculation of heat-killed *Str. agalactiae*

Reaction to living organisms

Our experience of mastitis in the goat, as produced by inoculation of *Str. agalactiae* into the teat canal, has led us to conclude that this experimental disease does not differ significantly from naturally occurring bovine streptococcal mastitis. This conclusion is based on clinical, bacteriological, biochemical and histopathological observations on some 50 experimentally infected animals. For this reason, the following remarks on the disease in goats have been written from a relative point of view, present knowledge of the disease in cattle being the basis for comment and comparison. Reactions to living organisms could be classified as (*a*) marked (*b*) intermediate (*c*) minimal. Table 1 summarises the reactions of animals in these groups and Figures 2 and 3 give complete data for typical marked and minimal reactions.

Temperature reactions. The occurrence of a raised body temperature following inoculation of streptococci into the teat canal of goats appears to be clinical evidence of activity of the organisms within the udder. It has been correlated with a high viable count of organisms per ml. of milk and sometimes with an alteration in mouse-lethal power of the organisms (Holman, Pattison and Taylor, 1950); moreover, it has been possible to cultivate the organism from the supramammary lymphatic gland of the inoculated side of the udders of goats destroyed at this time *vide infra*. All animals that have shown marked clinical abnormality of the udder at a later stage of the disease have previously shown one or more temperature reactions. The rise in temperature may be quite transitory, so that it would pass undetected unless readings were

TABLE I

GENERAL REACTIONS RECORDED FOR INOCULATED GOATS AND FOR MILK FROM INOCULATED SIDES.

	Marked reactions	*Intermediate reactions*	*Minimal reactions*
Number of goats	5	2	3
Temperature reactions	Began in 18 to 48 hrs. ; irregular high temps. over 12 days ; one, over 21 days.	Began within 48 hrs.; one, irregular high temps. over 11 days.	Two began within 48 hrs. with second rise on 8th day; one, abnormal on 19th day only.
Clinical condition of inoculated side of udder.	Swelling and hardness increasing for 14 days, then declining rapidly for 14 days to leave affected side very small but quite soft.	Swelling and hardness increasing over 14 days, then gradual reduction until about half preinoculation size.	Variable and slight ; no marked reduction in size.
Milk yield	Marked fall to less than 50 ml. at end of 26 days ; no recovery	Rapid fall to about half preinoculation level ; no recovery.	Two, yield down by about 200 ml. and remained thus ; one, only transient decrease.
Chlorides in milk	Increased throughout : two exceptions in one goat.	One, up for 10 weeks ; one, variable.	Variable ; sometimes less than uninoculated side.
pH of Milk	Variable for 4 weeks, then more alkaline than uninfected side.	Same or slightly more alkaline than uninfected side.	Variable.
Whiteside test	Four, positive throughout ; one negative for 4 weeks.	Positive for first 4 weeks.	A few positive readings.
Sediment volumes	Irregularly abnormal with highest readings between 1 and 4 weeks.	One, irregularly positive with highest readings between 1 and 3 weeks; one, slightly abnormal at 1 week.	Abnormalities slight and few.
Blood leucocyte counts	Transient leucopenia in 4 cases.	Transient leucopenia.	Within normal range.
Leucocytes in Breed smears ...	Many neutrophils present throughout.	Many neutrophils present throughout.	Many neutrophils present throughout.
Str. agalactiae per ml. of milk ...	High counts when temps. high ; counts decreased as disease progressed ; lowest count 200 per ml. ; one negative culture at 8 weeks, positive on following day.	One, all counts above 25,000 per ml. ; one, counts variable, lowest 700 per ml.	Two, all counts over 20,000 per ml. ; one, all counts over 1,000 per ml. ; in general, counts as high or higher than in marked reaction goats.

made at least twice daily, and it may be quite small, so that it might be unsuspected if pre-inoculation readings were not made for all individuals over several days. The mammary gland is apt to be regarded as an anatomically isolated structure, but our observations lend support to the view that an inflammatory pro-

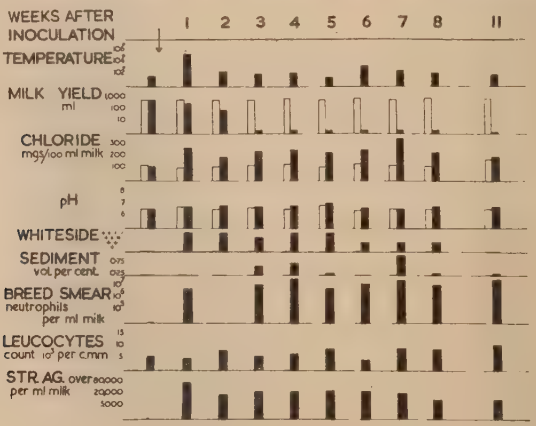


Fig. 2. Typical marked reaction to intramammary inoculation of living *Str. agalactiae*

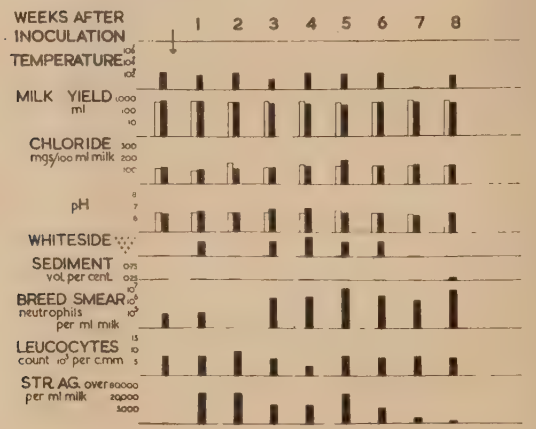


Fig. 3. Typical minimal reaction to intramammary inoculation of living *Str. agalactiae*

cess within this gland always involves a response from the body as a whole which may be marked, moderate, or slight.

Clinical condition of the inoculated side of the udder. In goats the typical reaction to infection with *Str. agalactiae* may be divided on clinical and histopathological grounds into three stages: (*a*) infection and initial systemic reaction, characterised histologically

by the accumulation of neutrophils in the secreting acini; (*b*) swelling and induration, characterised by accumulation of macrophages and fibroblasts in inter-acinar tissue, stasis of secretion, damage to secreting epithelium and obliteration of many secreting acini; (*c*) atrophy, characterised by interlobular and intralobular fibrosis and by lobular involution (Fig. 4). In cases of bovine streptococcal mastitis, Spencer and McNutt (1950) found that clinical induration of the udder was not associated with fibrosis; the fibrosed udder was small but soft. The same authors found inflammatory lesions in 13 of 19 carrier quarters and suggested that more intensive histological examinations might have disclosed lesions in the remaining six quarters. Our findings in goats confirm these observations.

Fig. 4

There are marked individual variations in the progress of the disease pattern, but our opinion is that only confusion can result from complex clinical descriptions of what is fundamentally one condition. Our experience has been that the presence of *Str. agalactiae* in the udder of the goat is evidence of an inflammatory process, be it ever so slight, and that all udders from which *Str. agalactiae* can be regularly isolated should be considered affected with streptococcal mastitis.

Milk yield. Measurement of milk yield is a delicate test for the presence of an inflammatory process, however slight, within the udder—provided always that the normal yield of any individual under experiment is well established and that care is taken to avoid those numerous extraneous factors, e.g. change of food or of milking technique, that can so easily alter the normal volume of

milk secreted within a stated period. An active inflammatory process always reduced the amount of milk secreted and a rise following a period of reduced yield was evidence of suppression of inflammatory activity. Histological findings supported clinical evidence that reduced milk yield was due to destruction of secreting elements. The udder that had shown extensive reduction in yield was characterised histologically by many fibrosed and involuted lobules and in udders less severely affected the amount of non-functional tissue was proportionately less; in some cases where the yield during life was only slightly below the pre-inoculation level, a fairly extensive histological search was necessary to establish the presence of non-functional tissue.

Chlorides, pH, sediment volume, and Whiteside tests. These tests measure the chemical changes in milk during the inflammatory process. Our experience has been that their value is limited because they do not detect slight inflammatory activity within the udder. When they were strongly positive the case was so obviously one of clinical mastitis that they did little but confirm conclusions that were more easily reached by other means. These results agree with those of Bryan (1946) who found that chloride percentage and pH tests and leucocyte counts of milk were of very little value in detecting the presence of udder infection in goats. The greatest sediment volume readings were obtained during the most extensive phase of the inflammatory process, at a time when clinical examination left no doubt that the udder was diseased.

Leucocytes in peripheral blood. If carried out sufficiently frequently, enumeration of blood leucocytes could be relied on to detect the first systemic reaction to inoculation—the phase of leucocyte migration from blood vessels to secreting acini—but after recovery from this leucopenia further changes in the udder were not obviously reflected in the peripheral blood.

Leucocytes in Breed smears. Milk from goats in full lactation was found to contain very small numbers of intact leucocytes. Milk from animals inoculated with *Str. agalactiae* usually contained grossly abnormal numbers of cells. There appeared to be some correlation between the cell count in milk and the extent of the inflammatory process. The milk from one goat inoculated with heat-killed organisms showed a transitory rise in leucocyte content. This test was the most sensitive and reliable measure of abnormality of the secretory elements of the udder.

Str. agalactiae *per ml. of milk.* The count of streptococci per ml. of milk was not invariably a measure of the intensity of the inflammatory process. A high body temperature was always accompanied by a high count of organisms in the milk, but high counts were also recorded at times when the temperature was normal. There was a general tendency for the count to be high during extensive involvement of tissue in the inflammatory reaction and then to fall during the processes of involution and fibrosis;

counts from atrophied udders were always low. By contrast, counts were usually relatively high when the inflammatory process was slight and long-continued. Negative cultures were obtained on only three occasions out of many hundreds of milk samples examined; two of these negative samples were taken six hours after inoculation with the delayed-action inoculum, and the other, eight weeks after the same type of inoculum; subsequent milk samples from these three goats contained *Str. agalactiae*.

Spencer and Angevine (1950) have expressed surprise that severe lesions in bovine mastitis can co-exist with low streptococcal counts in the tissues and milk. Our experience has been that a low count is rather typical of a late stage of the disease and it seems to us unnecessary to postulate the activity of secondary invaders or of trauma to explain widespread destruction of tissue by *Str. agalactiae*. We believe this streptococcus to be a pathogen in its own right but not one that will necessarily destroy all secreting tissue.

Bacteriological Examination of Supramammary Lymphatic Glands

A constant *post-mortem* finding was enlargement of the supramammary lymphatic gland of the inoculated side of the udder when compared with the gland from the uninoculated side (Fig. 5);

Fig. 5

in the early stages of the disease the gland from the inoculated side was obviously oedematous. The areolar tissue between the inoculated side of the udder and the abdominal wall was frequently gelatinous and the blood vessels of this area hyperaemic.

The majority of lymphatic glands were fixed *in situ* for histo-

TABLE II

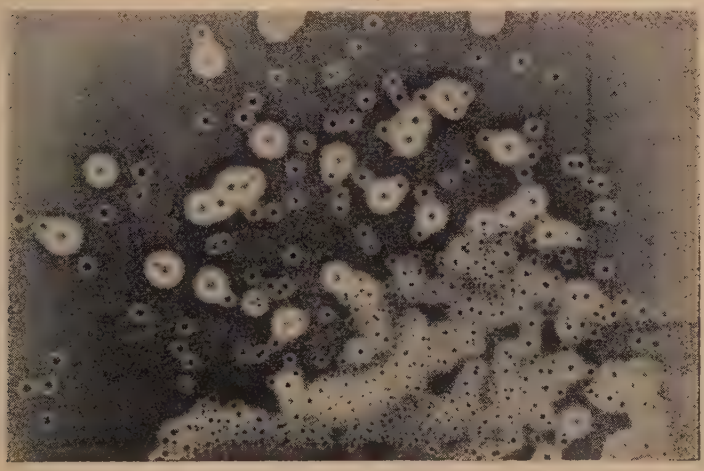
RESULTS OF BACTERIOLOGICAL EXAMINATION OF SUPRAMAMMARY LYMPHATIC GLANDS FROM GOATS INOCULATED INTO THE TEAT CANAL OF ONE SIDE OF THE UDDER WITH LIVING STRAIN S13 STREPTOCOCCI.

Goat No.	*Type of reaction to inoculation*	*Time between inoculation and destruction*	*Temperature reaction at death*	*No. of colonies of* Str. agalactiae *from :—*	
				Inoculated side	*Uninoculated side*
1	Delayed	6 hours	No	0	0
2	,,	3 days	No	1	0
3	,,	4 days	Yes	113	0
4	,,	7 days	No	0	0
5	,,	13 days	No	0	0
6	,,	13 days	No	0	0
7	,,	15 days	No	0	0
8	,,	15 days	No	0	0
9	,,	80 days	No	0	0
10	Rapid	6 hours	Yes	75	0
11	,,	24 hours	No	0	0
12	,,	2 days	Yes	1	0
13	,,	21 days	No	0	0

logical examination and could not therefore be cultured, but glands from inoculated sides of udders were removed from 13 goats before fixation of the rest of the tissue and were cultured for streptococci by removing a piece of tissue from the centre of the gland with aseptic precautions and rubbing this over the surface of a Bactotryptose ox-blood agar plate. Plates were examined for growth after 24 hours' incubation at 37° C.

Table 2 gives particulars of the goats from which glands were cultured. *Str. agalactiae* was identified biochemically and serologically. It will be noted that the inoculated organism was isolated from four different animals from the inoculated side only, but that it was never present in large numbers. Three of the four animals from which the organism was isolated had an abnormally high temperature at the time of death. It appears, therefore, that *Str. agalactiae* can enter the local lymphatic vessels at certain stages of the disease and that at these times there may be a general body reaction as evidenced by a rise in temperature. Histological examination of glands from which streptococci had been cultured confirmed their presence.

FIG. 6



Observation on a Variant of Strain S 13 Obtained after Growth in the Udder of Goats.

In two goats inoculated by the delayed reaction method, a colony variant of strain S 13 appeared in considerable numbers on the third day after inoculation in one case and on the fourth day in the other. This variant formed a darker colony on the surface of Bactotryptose ox-blood agar and had a narrower haemolytic zone than the strain as originally inoculated (fig. 6). After its first appearance, the variant was present in all milk cultures from these two goats until the animals were destroyed (two cultures in one

case and 13 in the other). It so happened that *Str. agalactiae* was obtained after death from the supramammary lymphatic gland of the inoculated side of one of these two goats (table 2, goat no. 3), and in the culture from this gland the dark colony variant was present in considerable numbers. From time to time occasional dark colonies of a similar type have been noted in milk cultures from various other goats.

DISCUSSION

The conclusion reached from our observations on experimental streptococcal mastitis in goats is that this disease does not differ significantly from naturally occurring bovine streptococcal mastitis associated with *Str. agalactiae*. Histopathological changes in goats (to be published later) were closely similar to those reported in cases of streptococcal mastitis in the cow (see Spencer and McNutt, 1950) and the clinical condition of the udder and biochemical changes in the milk were indistinguishable in both diseases.

As these disease processes were so similar it seems likely that the results of chemotherapeutic, immunological, bacteriological and pathological experiments on streptococcal mastitis in goats would be applicable to the cow.

SUMMARY

It was found possible to control the speed of reaction to infection of goats inoculated into the teat canal with living strain S 13 streptococci. The speed of reaction apparently depended on the number of living organisms inoculated, and a delayed reaction followed inoculation of about 1,000 living organisms contained in 3·0 ml. of saline and 7·0 ml. of Todd-Hewitt broth.

The course of the disease in goats was studied up to a period of 11 weeks after inoculation. No indirect test was completely reliable in detecting infection, but that found most sensitive was the leucocyte count in milk.

Bacteriological examination after death of experimentally infected goats revealed the presence of *Str. agalactiae* in the supramammary lymphatic glands of inoculated sides of udders in four out of 13 cases. In three of the four positive cases the animal had an abnormally high temperature when destroyed.

A dark colony variant of the strain of *Str. agalactiae* inoculated into the teat canal of goats was isolated in considerable numbers from the milk of two animals.

Examinations of the udder and tests of the milk over a period of 11 weeks, failed to reveal any essential difference between the experimental condition caused by inoculation of *Str. agalactiae* into the teat canal of goats and naturally occurring bovine streptococcal mastitis as described in the literature. It is suggested, therefore, that this experimental condition in goats could be used for the elucidation of many problems concerned with bovine streptococcal mastitis.

ACKNOWLEDGEMENT

We wish to express our thanks to Mr. F. H. Summerfield, Senior Technician of this laboratory, for preparing the photographs.

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SOME ABNORMALITIES OF KERATINIZATION IN THE SKIN OF SHEEP

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INTRODUCTION

A recent paper by Burns (1949) described the mass shedding of fibres from primary and secondary follicles which occurred in a Romney lamb between 3 and 6 months of age, as was shown by the presence of numerous empty follicles at the level of the sebaceous glands of the primary follicles, at which level follicle population counts were made. Data from the follicle population counts indicated a decline in the relative numbers of secondary follicles (S/P ratio) commencing prior to 6 months old and becoming accentuated by 9 months of age. This suggested the possibility that some of the secondary follicles had degenerated and disappeared. The health of the lamb was adversely affected by an attack of "foot-rot" during the period when shedding occurred.

A more detailed histological and cytological examination of the sectioned and stained material has since been undertaken, and this has revealed that many of the follicles show marked abnormalities in which they differ from typical shedding follicles.

Typical fibre-shedding in different mammals has been described in detail by several authors, such as Segall (1918). A certain stage in this process, in which the proximal end of the cortex appears to be split up in brush fashion and interlocks with the tissue of the follicle, is referred to for convenience as the "brush" stage and this type of shedding is called "brush-shedding".

The abnormalities may be grouped into three types which are described below. In order to simplify description, the regions of the follicle at which the different phases described occur are indicated by Roman numerals on the accompanying diagrams (Figs. 1 and 2).

TYPES OF FOLLICLES

The "Plugged" Type

The proximal portions of degenerating follicles of this type show some resemblance to follicles in which the fibre has developed a "brush" end and is about to be shed in a typical fashion (see Fig. 1). In such follicles the papilla appears approximately spherical and incompletely enclosed by a thin epithelial layer, suggesting comparison with an egg in an egg-cup. The solid proximal portion of a typically shedding follicle (Pl. IId) immediately above its papilla consists of cells with solid cytoplasm and large ovoid nuclei. However, in the "plugged" degenerating follicles this region (Pl. Ia, b) consists of cells with deformed nuclei and obliterated cytoplasm

rich in glycogen, i.e. of the same type of tissue which generally forms the outer root-sheath within the proximal half of normal follicles with growing fibres. The most proximal region (I) of the follicle consists entirely of a uniform plug (Pl. Ia) of this tissue. Hence this type of follicle is termed "plugged". Immediately above the papilla the axial portion of this plug sometimes forms a short conical region which is composed of smaller cell-bodies (Pl. Ib), and thus shows the nuclei more closely arranged, than in the rest of the plug.

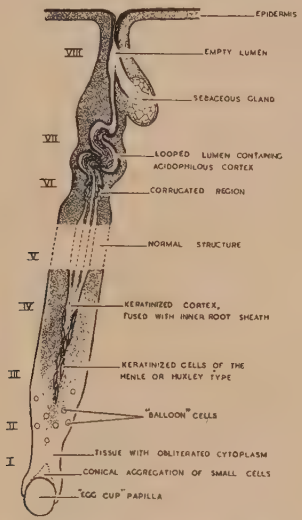


FIG. 1.—Diagrammatic longitudinal section through a "plugged" follicle.

In region II the axial cells become solid and elongated, with a strand of keratinized elements of the type of Henle or Huxley cells becoming visible in the axial portion of region III (Pl. Ic). Within the peripheral cell-layers of the regions II and III a few scattered solid cells with refringent nuclei of a type evidently corresponding to the small "balloon" cells described by Unna (1900) can be made out. Similar cells were also observed by Branca (1899) in regenerating epidermis of *Triton*. Other cells, showing "reticular" degeneration (cf. Unna, 1900), can be observed in the vicinity of the "balloon" cells.

In region IV the axial structure, consisting of keratinized cells of the type seen in the layers of Henle and Huxley, widens and encloses a picrophil structure of the appearance of fully-keratinized cortex (Pl. Id). This structure is often firmly fused with the basophil Henle or Huxley elements which do not here permit distinction of the layers seen in normal follicles.

Region V commonly resembles normal follicles at the level at which the cortex as well as all three layers of the inner root-sheath

are keratinized, both cuticles being present. In region VI de-keratinization and re-absorption of the inner root-sheath may take place on the surface of a series of corrugations and immediately below them, as described by Auber (1951) for normal follicles from sheep.

In region VII the cortex, which has been found in a fully-keratinized state at deeper levels, undergoes peculiar changes (Pl. Ie, f; Pl. IIa). Large, often irregularly outlined portions of it stand out by their acidophil staining reaction and often enclose an irregularly and indistinctly outlined axial portion. This is picrophil in reaction and thus apparently normal. The acidophil portions show a distinct striation, indicating fibrillary structures, and have marked cell-boundaries and an empty space in the centre of each cell in the original position of the nucleus. These lengths of atypical cortical tissue are generally covered by continuous portions of the fibre cuticle (Pl. If) which retains here its normal texture and the staining property typical of its fully-keratinized state. However, these sheets of the fibre cuticle are always delaminated from the cortical portions.

In region VII (Pl. If; Pl. IIa) the lumen of the follicle narrows and takes a peculiarly looped course either within a comparatively massive portion of the follicle, or the follicle is thin and shows the same bent or looped shape as the lumen. The loops of the latter may lie in various planes and in one transverse section of the follicle several of the loops may be cut. It is within this bent lumen that the cortex completely changes into the abnormal acidophil state described above. The fibre cuticle eventually disappears. The cortex adheres to the lining of the lumen, along which it gradually becomes thinner and finally disappears. The lumen finally appears empty and much compressed. The lining of the lumen, although for its greatest part showing the acidophil staining properties of normal stratum corneum, does not show a laminated texture. It is more or less homogeneous and slightly refringent. Immediately beneath the lining many cells of the follicle wall contain keratohyalin granules.

In region VIII from somewhat below the level of the sebaceous gland up to the skin surface the lumen straightens again and becomes lined or blocked with loosely laminated stratum corneum as in the corresponding region of a follicle from which the fibre has been shed normally. Sometimes frayed portions of acidophil cortical tissue may project into the lumen of this region.

Variations

Certain variations from the above type of degeneration can be observed as follows:

(*a*) No portion of the follicle shows normal features; the state obtaining in region IV changes directly to that in region VII.

(*b*) The conditions within the regions III and IV may be more elaborate, with the cells of the Henle and Huxley type remaining

separated and forming actual concentric layers (Pl. IIb). Some of the cells which apparently belong to Huxley's layer may also contain trichohyalin. However, the axial tissue may show some of the features of the degenerating cortex seen at more proximal levels of "lymphatic" follicle described below (p.42): it may present a more or less homogeneous and vacuolized state.

(*c*) In one follicle, regions II and III show traces of the "brush" end (Pl. IIc), as seen in typically shedding follicles. In region V this state passes into one in which all layers of the inner root-sheath and the fibre can be made out. Moreover, in the lower portion of region V the cells of the root-sheath cuticle and of the fibre cuticle are flattened (as is generally found on shedding fibres immediately above the "brush" end) and not imbricated. In regions VI to VIII the features described above for the usual "plugged" follicles are found.

The "Lymphatic" Type

In region I this type shows a bulb and a papilla of the outlines seen in normal follicles with growing fibres (see Fig. 2). However, the tissue of the wall of the bulb appears disrupted by extensive

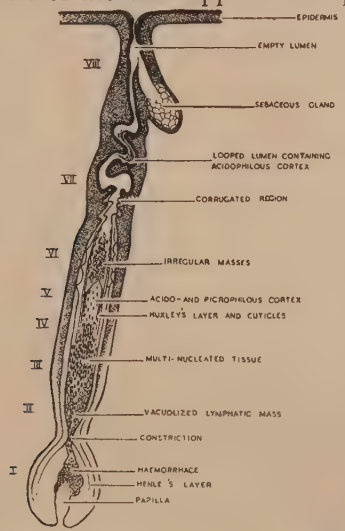


FIG. 2.—Diagrammatic longitudinal section through a "lymphatic" follicle.
(For diagrammatic purposes the follicles, although actually bent in a complicated fashion, are represented straight and somewhat foreshortened, without respect to the relative length of the different regions indicated in Roman numerals.
Stratum corneum (typical and modified) is indicated in solid black.)

haemorrhages (Pl. IIe) which are generally in contact with the papilla and contain either closely-packed haematocytes at different stages of degeneration or a more or less homogeneous lymphatic mass with a few scattered large cells. As a rule, Henle's layer is the only one which appears outlined within the bulb and remains

the only distinct layer for a considerable distance further distally; it is wider than in normal follicles.

At the transition from region I into region II the lumen of the follicle is constricted, without any reduction in the width of Henle's layer. In some follicles the lymphatic mass extends through the constriction and into region II, and becomes strongly vacuolized (Pl. IIf). In other instances the contents of the lumen at and above the constriction, i.e. in region II, approaches in appearance the "multi-nucleated" tissue described below (region III). The process of transition from the lymphatic mass to the "multi-nucleated" tissue varies in detail and position in different follicles.

Region III contains an axial structure which differs from typical cortical tissue at the corresponding level by having a much greater number of smaller and more densely arranged nuclei (Pl. IIf); these, however, show the typical elongation parallel to the main axis of the follicle. The cytoplasm of this tissue seldom shows any fibrillation or cell-boundaries. Henle's layer is often seen to contain trichohyalin in this region, up to a much more distal point than in normal follicles.

In region IV the other layers of the inner root-sheath and the fibre cuticle may be formed in succession by delamination from the above-described "multi-nucleated" axial tissue, with Huxley's layer developing a smaller amount of trichohyalin droplets than in the normal state.

Towards region V the "multi-nucleated" tissue may pass in a patchy manner into a picrophil substance of the outline of fully-keratinized cortex. This transition may even take place through a short phase in which fibrillary structures can be made out within acidophil cytoplasm and which is thus almost identical with normal cortical tissue prior to its keratinization. In most instances, however, such a transitional acidophil region contains more densely arranged nuclear remnants than the corresponding normal tissue, and some of these remnants even persist within the more distally situated picrophil substance (Pl. IIIa). Normal fully-keratinized cortex contains no such nuclear remnants.

Within region VI this transitional phase passes into a state in which the inner root-sheath encloses a wide lumen, containing irregular masses lying loosely within it. These masses are mostly rounded (Pl. IIIb), often of rather bizarre outlines (Ph. IIc) but here and there also of narrow, prismatic shapes. They are refringent, of homogeneous texture and chiefly picro-, but often acidophilic. The inner root-sheath is here either still keratinized or passes into the state of de-keratinization.

The features within region VII are analogous to those seen in regions VI and VII of "plugged" follicles, with a more or less distinct corrugated region passing distally into a portion with a looped lumen. Generally the lumen at the level of the corrugations is narrow and empty, with the above-mentioned irregular masses rarely projecting so far distally. Within the looped portion of the

lumen short lengths of the abnormal, acidophil cortex described above may occur.

In region VIII the same features as in the corresponding region of the "plugged" type are found.

Variations

The "lymphatic" type varies as follows

(*a*) The axial structure does not become picrophilic at any level but, before disappearing within region VII, attains again the lymphatic, more or less vacuolized state found further below in region II and displays a few large but unorientated nuclei. This state may be associated with the lack of Huxley's layer and the cuticles.

(*b*) The lumen in region VII is straight and reduced to a narrow slit, with the wall of the follicle also thinning down.

The "lymphatic" type of degeneration is more frequent in samples taken from the lamb at 9 than at 6 months old.

The "Vesiculated" Type

The very few follicles of this striking type found at 9 months show the outline of the bulb and haemorrhages within it (Pl. IIId) exactly as described for region I of "lymphatic" follicles. However, not even Henle's layer becomes delineated within the bulb.

For some distance above the bulb no lumen or axial structure is present and the cytoplasm shows the greatly obliterated state and rich glycogen content (Pl. IIId) seen at corresponding levels in "plugged" follicles.

Somewhat further distally the cytoplasm first becomes solid, with some of the cells showing "ballooning" (p.39). Some of the "balloon" cells have become detached from the bulk of the epithelium and entered the surrounding connective tissue. More distally the solid epithelium becomes disrupted by a series of proximo-distally arranged spherical vesicles lined by flattened epithelial cells (Pl. IIIe). These vesicles contain some small "balloon" cells, masses of an acidophil granular substance, and some degenerated haematocytes. Outside these vesicles, where the epithelial cells are still interconnected, the intercellular bridges appear elongated, with leucocytes being forced here and there into the distended intercellular lymphatic spaces.

Above this "vesiculated" region a wide axial strand is formed by disarranged elements of the layers of the inner root-sheath, partly containing trichohyalin and partly keratinized. At one point this axial strand encloses a few elongated fibrillary cells which show features of cortical tissue prior to keratinization. At a somewhat higher level the elements of the inner root-sheath fuse into layers which enclose a collapsed and folded empty lumen.

Near the skin surface, where the duct of the sebaceous gland opens into the follicle, a wide space is formed round the layers of the inner root-sheath which are firmly enclosed by a thin layer of

more or less laminated stratum corneum (Pl. IIIf). This layer appears to be detached from the epithelial follicle wall by sebaceous secretions.

In the most distal region of the follicle the layers of the inner root-sheath break up into their single keratinized elements of a typical, angular shape. Although their cover of stratum corneum cells completely disintegrates at this level, these cells of the inner root-sheath still form a coherent structure. This lies loosely within the very wide follicle lumen which is lined with normal stratum corneum.

DISCUSSION

Among the features common to the "plugged" and the "lymphatic" follicles the changes in the cortex are particularly remarkable. The distinctness of the fibrillary structures, in conjunction with the presence of empty nuclear spaces, renders this atypical cortex very similar to the state in the enzyme-treated fibres described by Wildman (1947) and other authors.

In addition to these features two other facts must be taken into consideration, namely that (*a*) such lengths of abnormal cortex only occur above the upper limit of the inner root-sheath, and (*b*) apparently normal, picrophil portions are frequently present within the axial region of these lengths.

As pointed out by Auber (1951), in normal follicles from sheep keratinization of the cortex proceeds from a point on the periphery and never from the axis of the fibre. The inner root-sheath at its upper limit, i.e. on the surface of the corrugated region, becomes disposed of by de-keratinization followed by re-absorption, apparently owing to some enzymatic action.

From these details it appears probable that the abnormal cortex under discussion is secondarily de-keratinized and not inhibited in its keratinization. The de-keratinizing enzymatic agent, the action of which is normally restricted to the inner root-sheath, may here have "run wild", extending its activity to the cortex and affecting first the peripheral and later also the axial portions of this layer. If this hypothesis is correct the fibre cuticle, which remains intact and picrophilic, must be resistant to the enzyme; yet the enzyme must be able to pass through the cuticular covering and reach the cortex of the fibre.

That, in principle, de-keratinization is possible within the follicle has been assumed by Segall (1918) who postulates re-absorption of the processes of the "brushes" in shedding fibres. The authors of the present paper have, in fact, recently observed that the "club" ends of fibres from a Romney ram, which lie loosely in the most distal portion of the follicle lumen, have their superficial layers transformed into an acidophil, probably de-keratinized substance.

In the "lymphatic" type the conditions in the "multinucleated" cortical tissue described above may indicate a similar

state of degeneration to that found by Unna (1900) in his "balloon" cells. However, actual "balloon" cells have not been observed in "lymphatic" but only in "plugged" and in "vesiculated" follicles. According to Unna, these cells do not contain fibrillae but stain like fibrin; this suggests that the substance which normally forms the fibrillae is suspended within the colloidal system of the "balloon" cell.

The presence of the fibrillary substance in a similarly non-orientated state in the "multi-nucleated" tissue appears to be the cause of the bizarre shapes which in "lymphatic" follicles partly or entirely replace normal fully-keratinized cortex. It is suggested that the process of keratinization, instead of transforming the normal, parallel-orientated fibrillary structures (see Auber, 1951), may here precipitate the same substance from its atypical colloidal suspension into irregular, massive aggregates.

Lack or incompleteness of the fibrillary system also within the wall of the bulb may lower the mechanical resistance of the tissue of the latter and thus be the cause of the haemorrhages seen there.

In the "vesiculated" type the presence of "balloon" cells, oedematous infiltration of leucocytes into the intercellular spaces and the formation of vesicles definitely suggest a process of acute inflammation; it is comparable to the degeneration found in Herpes and similar conditions, but no micro-organisms could be made out and the epidermis proper appears normal. The vesicles found in the follicles under discussion also appear to agree with Branca's (1899) description of the "abscès en miniature" observed by him in regenerating epidermis of the Axolotl. The inflammation of the follicles appears to have proceeded in a proximo-distal direction, as indicated by the presence of keratinized inner root-sheath lying loosely within the lumen at abnormally high levels, as if about to be extruded.

It remains to discuss the possible causes of the wide-spread degeneration of follicles in this sheep. In the follicle population counts (Burns, 1949) made at the level of the sebaceous glands of the primary follicles, all empty follicles were counted as "shed". It is clear that such follicles had indeed lost their fibres, but the evidence here presented indicates that in the majority of the follicles the normal process of "brush-shedding" had not occurred, unless it occurred and was followed by partial and abnormal re-growth of the fibre. As pointed out above, the "plugged" type of degeneration has some features in common with "brush-shedding" follicles, and this similarity appears to be emphasized by the single follicle described under (*c*). The "plugged" follicles, however, contain large quantities of glycogen, whereas "brush-shedding" follicles of sheep contain much less of this substance than is present in follicles with growing fibres. Brunner (1907) states that shedding follicles of man do not contain any glycogen. It is possible that the "plugged" type may be the result of a deviation

from the "normal" process of "brush-shedding" in its earliest phase and prior to the utilization of the glycogen, but it may be an unrelated process.

It is a curious fact that the three types of degeneration described, although occurring together in the same material, show no clear relationship with each other, such as might indicate them to be different stages in a progressive process. The "vesicular" type, although combining some characters of the "plugged" and the "lymphatic" types, is too divergent in many respects to be considered intermediate between them, and very few other follicles were found which could conceivably be considered intermediate.

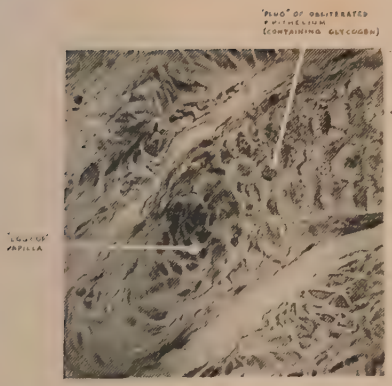
The rarity of intermediate types is not, of course, a decisive argument against the "plugged" and "lymphatic" types being part of the same process, since the long period of three months elapsed between sampling dates, and in any case there might be no gradual transition from one stage to the other. If, as suggested above, the "plugged" type is the result of an early deviation from the "brush-shedding" process, then it may be that the bulb is reconstituted, as in normal follicles after shedding, but in an abnormal manner resulting in the "lymphatic" type. If this should be the case, the conical region consisting of small cells, which sometimes occurs immediately above the papilla of "plugged" follicles, may indicate the first stages of formation of an abnormal, "multi-nucleated" fibre.

However, the "lymphatic" type shows in its proximal parts greater similarity to a normal non-shedding follicle than does the "plugged" type. It is therefore not impossible that the "lymphatic" type is the earlier phase and the "plugged" type a further reduced condition, its resemblance to a "brush-shedding" follicle being incidental.

In view of the absence of a definite sequence and of the scarcity of intermediate stages, we cannot exclude the possibility that the three types of degeneration may represent alternative processes, and not successive stages of degeneration, although we can advance no reason for such an apparently illogical state of affairs. Unfortunately the animal was disposed of before these skin samples were examined and no further samples could therefore be obtained. By the time the final samples were taken, when the lamb was 9 months old, it appeared to have regained its health (see Burns, 1949) and was beginning to thrive. The presence of many apparently normal follicles, including some in which fibre replacement after shedding was taking place (the tip of the new fibre not yet reaching the skin surface) indicates that there was no general inhibition of normal follicle activity. No skin disease was manifest and there had been no myiasis nor other damage to the skin.

In regard to the question which initiated the present inquiry, as to the fate of the secondary follicles which the follicle population counts indicated to have been lost, no satisfactory additional in-

Plate I.



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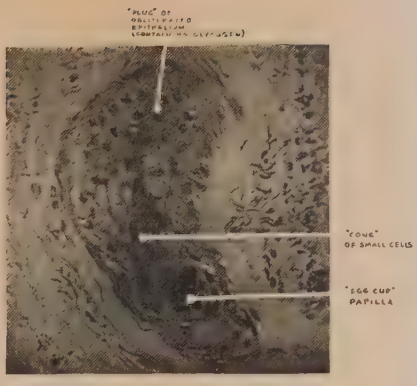

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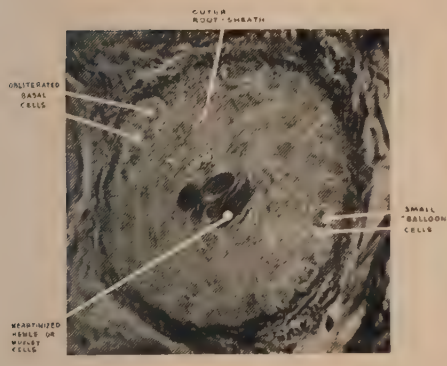

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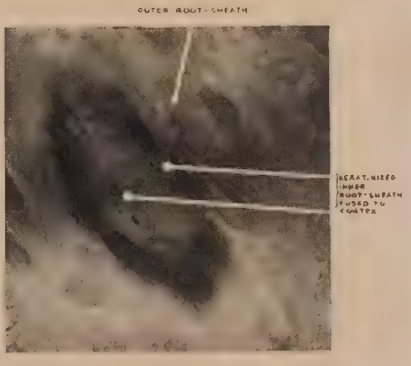

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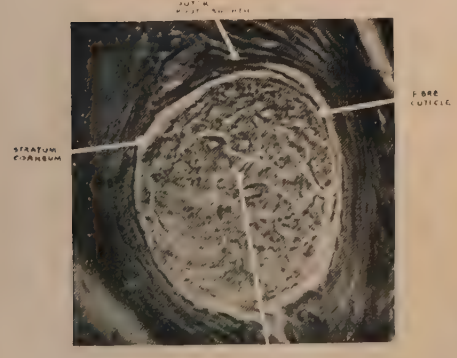
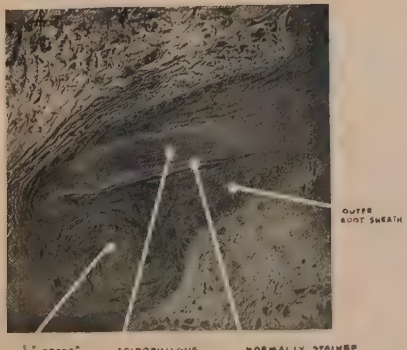


ACIDOPHILOUS CORTEX SHOWING CELL BOUNDARIES, TRANSVERSELY-CUT FIBRILLARY STRUCTURES AND NUCLEAR SPACES

(e)



(f)

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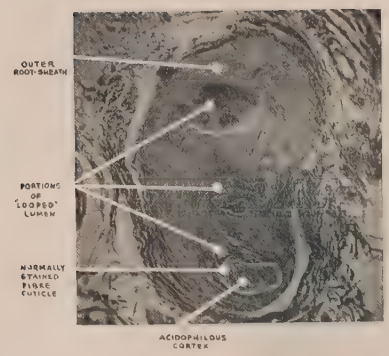

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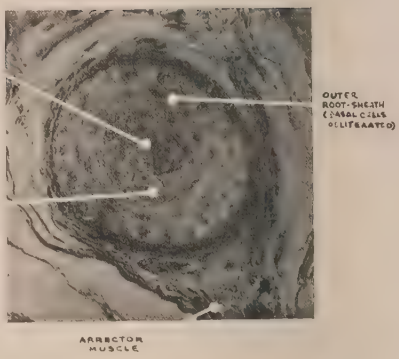

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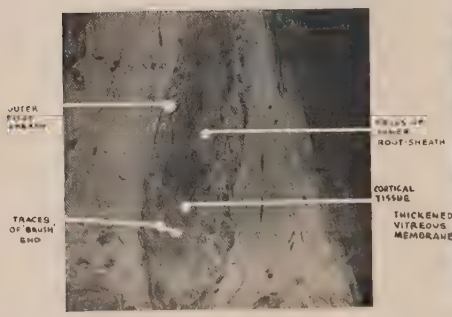

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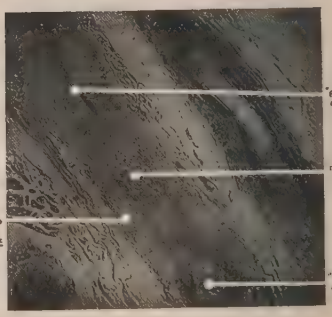

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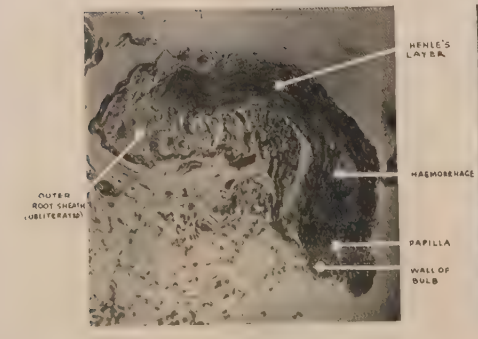

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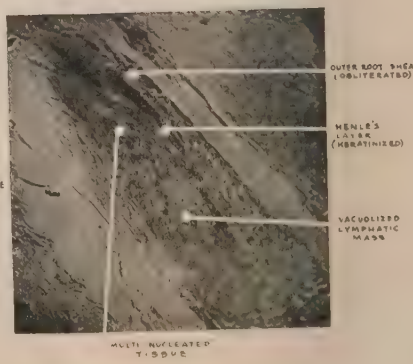

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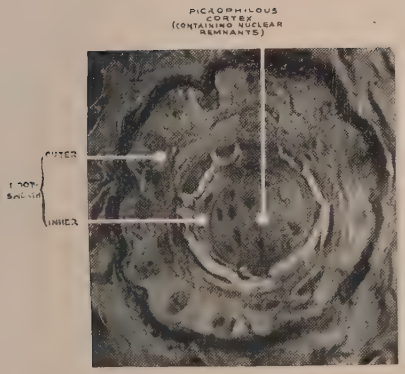

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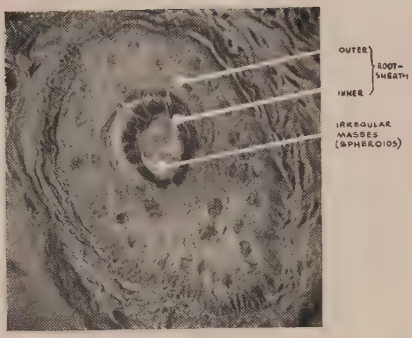

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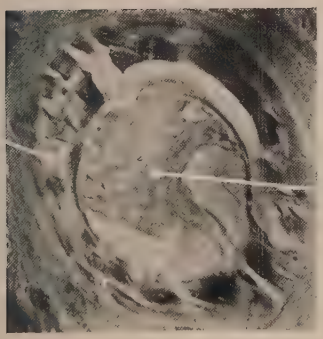

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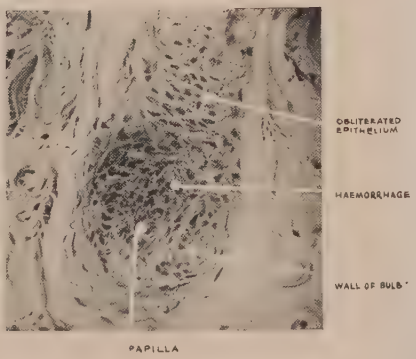

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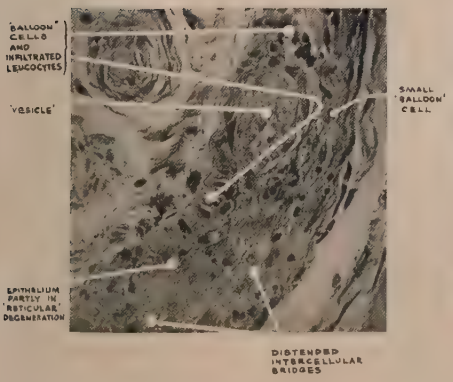

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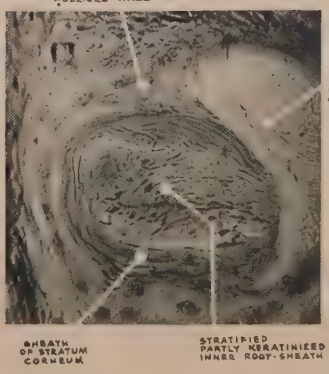
EPITHELIAL FOLLICLE WALL
SEBACEOUS DISTENSION OF LUMEN
SHEATH OF STRATUM CORNEUM
STRATIFIED PARTLY KERATINIZED INNER ROOT-SHEATH

(f)

Explanation of Plate

Pl. I.—" Plugged " follicles from Romney lamb aged 6 months (cf. Text Fig. 1) :—

(*a*) Region I in longitudinal section. (396x).

(*b*) Region I in longitudinal section. (277x).

(*c*) Region III in transverse section. (425x).

(*d*) Region IV in oblique section. (1108x).

(*e*) Region VII with portion of lumen in transverse section. Note the distinct cell-boundaries, fibrillary structures, and nuclear spaces of the de-keratinized cortex. (805x).

(*f*) Region VII with portion of lumen in longitudinal section. Note delamination of fibre cuticle from the de-keratinized cortex. (195x).

Pl. II.—(*a-d*) and (*f*) :—Follicles from Romney lamb aged 6 months. (*e*) :—from same lamb aged 9 months :—

(*a*) " Plugged " follicle. Region VII in transverse section, with lumen cut at three different points along its length (cf. Text Fig. 1). (189x).
same lamb aged 9 months :—

(*b*) " Plugged " follicle. Modification " b " of region III. (338x).

(*c*) " Plugged " follicle. Modification " c " of regions II and III. (226x).

(*d*) Typically " brush-shedding " follicle, for comparison with the " plugged " type. Proximal region in longitudinal section. (166x).

(*e*) " Lymphatic " follicle. Region I in longitudinal section (cf. Text Fig. 2). (254x).

(*f*) " Lymphatic " follicle. Regions II and III in longitudinal section. (307x).

Pl. III.—Follicles from Romney lamb aged 9 months :—

(*a*) " Lymphatic " follicle. Region V in transverse section (cf. Text Fig. 2). (590x).

(*b*) " Lymphatic " follicle. Region VI in transverse section. (422x).

(*c*) " Lymphatic " follicle. Region VI in transverse section. (805x).

(*d*) " Vesiculated " follicle. Bulb and adjoining region in longitudinal section. (246x).

(*e*) " Vesiculated " follicle. Middle region in longitudinal section. (308x).

(*f*) " Vesiculated " follicle. Distal region in transverse section. (347x).

formation has been obtained. In one instance only a "capsule", such as has been described in aged sheep (Burns and Clarkson, 1950), was found; all other degenerate follicles described reached the skin surface and would therefore have been included in the follicle counts. It should be noted, further, that the abnormalities described occurred in primary as well as in secondary follicles.

The detailed examination of the abnormal follicles here described has thus provided some rather tantalizing information which does not permit of any satisfactory interpretation at the present time, but which may be elucidated further by future studies on skin and follicle activities in the sheep.

SUMMARY

The skin of a Romney lamb from which widespread fibre-shedding had occurred was found to contain numerous abnormal degenerate follicles. Three types of degeneration are described which differ in their effects on the more proximal parts of the follicle. In many instances what appears to be secondary dekeratinization of the fibre cortex occurs near the skin surface, and none of the degenerate follicles contained a normal fibre.

The possible relations between the various types of follicle degeneration are discussed and it is concluded that the different types are not necessarily successive stages of one process.

No additional information has been obtained as to the fate of the secondary follicles which the follicle population counts indicated to have been lost.

ACKNOWLEDGMENTS

We wish to express our gratitude to Dr. A. B. Wildman for his kind encouragement and criticism, as well as to all our colleagues in the Biology Department, especially to Mrs. H. Clarkson, Miss D. Horner, and Miss C. Sykes for their help in technical matters, and to Mr. H. M. Appleyard and Miss C. M. Greville for preparing the photomicrographs.

Our thanks are due to Dr. A. B. D. Cassie and the Council of the Wool Industries Research Association for facilities to carry out this work and for permission to publish.

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STUDIES ON RESPIRATORY DISEASES OF PIGS.

I. MODIFIED HAEMAGGLUTINATION-INHIBITION TECHNIQUE FOR TITRATION OF INFLUENZAL ANTIBODIES IN PIG AND FERRET SERA.

By

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INTRODUCTION

Since virus haemagglutination was first described by Hirst (1941) and McClelland and Hare (1941), the haemagglutination-inhibition (H.I.) test has been widely used for titration of antibody against influenza in human and certain animal sera. When attempts were made in this laboratory to use the H.I. test for titrating antibody in swine sera against the Cambridge Strain (C1) of swine influenza, it was found that the test was of little value if the usual techniques were followed. This paper describes work which led to a modified test giving satisfactory results.

MATERIALS AND METHODS

Red cell suspensions. Red blood corpuscles (r.b.c.) from fowls or guinea pigs have generally been used, but occasionally also those from pigs. Packed normal r.b.c. which had previously been washed three times in normal saline, were stored at 4° C. up to a week. A 2 per cent. suspension in normal saline was made just before the test.

Strains of influenza virus. The strains C1 and Barratt were received from the World Influenza Centre by the courtesy of Dr. C. H. Andrewes, and strains 15 and PR8 were kindly supplied by Dr. J. A. Miles. Most of the experiments were done with the C1 strain of swine influenza, which was isolated at this laboratory by Blakemore and Gledhill (1941). For the present work, two lines of this strain were maintained, one by mouse passage and one by allantoic passage in chick embryos. The former was used for inoculation of pigs, and the latter for preparation of allantoic fluid antigen for H.I. tests.

Haemagglutination tests. The tests were performed in Dutch artist's tiles (Fig. 1). Mulder and Goslings (1948) have compared titrations in these tiles and in tubes; no significant difference was observed. Serial two-fold dilutions of virus in 0·1 ml. volumes were made in 0·85 per cent. saline. An equal volume of a 2 per cent. r.b.c. suspension was added and the tile was gently but thoroughly agitated to mix the fluids. The cells were allowed to settle at room temperature (19° C. to 20° C.). Readings were taken after half an hour if fowl r.b.c. were used, and after one hour if mammalian r.b.c.

* Under a grant from the Agricultural Research Council.

were used. The dilution showing definite but incomplete agglutination (+) was taken as the end point (see photograph 1).

Haemagglutination-inhibition tests. All sera were inactivated at 56° C. for 30 minutes. Serial two-fold dilutions were made with saline in 0·1 ml. volumes. An equal volume of the virus dilution containing four haemagglutinating doses (h.d.) was added to each of the serum dilutions. As a control, the virus dilution selected was titrated to give 4, 2, 1 and $\frac{1}{2}$ h.d. Finally, an equal volume of 2 per cent. r.b.c. suspension was added. If fowl r.b.c. were used, the readings were taken after 45 minutes, or if mammalian r.b.c. were used, after $1\frac{1}{4}$ hours, at room temperature.

Antisera. Pig and ferret sera against swine influenza were taken from pigs A2 to A7, and from one ferret, twelve days after experimental intranasal inoculation with strain C1.

CHOICE OF VIRUS ANTIGEN AND RED CELLS

Virus Antigen

In these tests infected allantoic fluids from chick embryos are usually used for titrations against influenzal antibodies in human and animal sera. Hirst (1942) reported haemagglutination with mouse lungs infected with influenza viruses, but he did not get haemagglutination with infected ferret lungs, due, apparently, to the presence of an inhibitory substance. In this laboratory, several types of antigen were tried, some of which were more suitable than others.

Pig lung suspension. A 1 in 5 suspension of lungs of a pig which died on the third day after an intranasal inoculation with C1 virus, was titrated with fowl and guinea pig r.b.c. The suspension agglutinated fowl r.b.c. but not guinea pig r.b.c. However, the agglutination of fowl r.b.c. was incomplete with the undiluted suspension even though some agglutination was observed up to a dilution of 1 in 16. It was hoped that the titre of the antigen might be improved by incubation Accordingly a portion of the original suspension was incubated for 1 hour and a second portion for 4 hours at 37° C. These incubated suspensions, however, did not agglutinate fowl or guinea pig r.b.c., even when used undiluted. This source of virus was abandoned as an antigen for the titration of pig sera.

Mouse lung suspensions. In order to determine the optimum period for the production of mouse lung antigen, mice were inoculated intranasally with infected mouse lungs of swine influenza virus strain C1, and were killed on the 2nd, 4th and 7th days after inoculation. Lungs of mice killed on each of these days were emulsified separately and a 1 in 5 suspension made. The suspension was lightly centrifuged and the supernatant titrated. The suspension of 2nd day lungs up to a dilution of 1 in 4 agglutinated fowl r.b.c., while those made from 4th and 7th day lungs agglutinated only when used undiluted. In another experiment, lungs of mice inoculated with a suspension of infective pig lung were used.

Suspensions of lungs (1 in 5) of mice killed on the 2nd and 7th day, and of those which died on the 5th day, were titrated. In this experiment also, higher haemagglutination titres were obtained with a suspension of lungs of mice killed on the 2nd day. It is evident, therefore, that the lungs of mice killed on the 2nd day were quite adequate and for practical purposes quite satisfactory; however, three days after this suspension had been stored at 4° C., the titres were reduced by 50 per cent. Unlike allantoic fluids from infected chick embryos the agglutination with stored mouse lung suspension was neither complete nor clear. If mouse lungs were to be used as a source of antigen, it would be necessary to prepare a fresh suspension on the day it was required. They were not adopted, therefore, for routine purposes.

Allantoic fluids. Chick embryos, which had been incubated at 37·5° C. to 37·8° C. for 11 days, were inoculated in the allantoic cavity with a 10^{-2} dilution of infected allantoic fluid by the technique described by Beveridge and Burnet (1946); the embryos were incubated at 35° C. for 48 hours after inoculation, chilled overnight at 4° C. and then harvested. The allantoic fluids gave titres varying from 40 to 320 with both fowl and guinea pig r.b.c. Bacteriologically sterile fluids giving titres of 80 and over, were pooled and stored at 4° C. The pool was used within 30 days of harvesting as virus antigen for routine tests.

Virus agglutination of r.b.c. of fowl and guinea pig.

Clark and Nagler (1943) showed that r.b.c. of several animal species were agglutinated by human influenza viruses and by strain 15 of swine influenza. Quantitative data were later reported by Chu (1948). The effect of swine influenza virus strain C1 on r.b.c. of fowl, pig and guinea pig was therefore investigated. This virus (C1) did not agglutinate r.b.c. from pigs when titrated either at room temperature, or at refrigeration temperatures (4° C. to 6° C.). With allantoic fluids, fowl and guinea pig r.b.c. gave approximately equal titres. These results are in agreement with those reported by Chu.

Most pig sera contained agglutinins against fowl r.b.c. It was therefore found that when fowl r.b.c. were used for H.I. tests with pig sera it was first necessary to adsorb out these agglutinins. This difficulty was not encountered in guinea pig r.b.c. as no pig sera were found to agglutinate these cells at or above a dilution of 1/10.

These results suggest that guinea pig r.b.c. could be used for titrating antibody in pig sera with strain C1. Nevertheless, since the use of fowl r.b.c. is so general, they were used in earlier experiments for haemagglutination-inhibition tests after agglutinins from pig sera had been adsorbed with fowl r.b.c. Ultimately, however, guinea pig r.b.c. had to be used for titration of pig sera to overcome non-specific inhibition which was encountered when fowl r.b.c. were used.

OCCURRENCE AND NON-SPECIFIC NATURE OF INHIBITION OF HAEMAGGLUTINATION BY NORMAL SERA.

Non-specific inhibition (N.S.I.) of haemagglutination by influenza viruses A and B with normal human, ferret, mouse, rabbit and guinea pig sera in low dilutions was first reported by Hirst (1942). McCrea (1946) noted that N.S.I. titres in normal human and animal sera varied greatly with the virus strain used for testing, the influenza A strain BEL D giving particularly high titres with most sera. Typical values for normal rabbit sera with this strain varied from 100 to 300. Burnet and McCrea (1946) reported N.S.I. titres of 300 to 1200 in sera of a number of normal ferrets.

N.S.I. against swine influenza virus strain C1 was encountered in sera of apparently normal pigs. The inhibition titres were the same (160) with most of the pig sera. Similar titres of N.S.I. were also encountered with sera of one normal horse and three normal rabbits, but titres of less than 10 were observed with sera of five normal cattle. Sera of more than 20 normal ferrets were tested, and all showed N.S.I. titres of 200 to 800.

The non-specific nature of the inhibition was confirmed by neutralisation tests in mice. Serum of pig A5 taken before, and 12 days after inoculation with swine influenza virus strain C1, and serum of a normal horse, were each mixed in a separate tube with equal volumes of a 1 in 100 broth suspension of infected lungs of mice killed 48 hours after inoculation (strain C1). The tubes were shaken well and kept at 4° C. for 2 hours. Each serum-virus mixture was inoculated intranasally into a group of mice. The results are shown in Table I.

TABLE I

NEUTRALISATION TESTS IN MICE

Sera	*4th day*	*7th day*
Normal horse	++++	++++ +++ +++ +++
Pre-inoculation Pig A5	++++ ++++	+++ +++
Post-inoculation Pig A5		— — — —

++++ = Mouse died with complete consolidation of lungs.
+++ = Mouse showed almost complete consolidation when killed.
— = Mouse killed and lungs found normal.

The non-specific nature of inhibition by the sera of normal pigs has since been confirmed by titration of the sera by the conglutinating complement absorption test (which will be described in a later paper).

AVOIDANCE OF NON-SPECIFIC INHIBITION IN NORMAL PIG SERA

Unsuccessful attempts.

Anderson, Burnet and Stone (1946) have shown that the N.S.I. titres are minimal if one uses the r.b.c. of a fowl selected on the ground that its r.b.c. give the highest titres in straight titrations of virus. Strains C1, PR8 and 15 gave highest titres with r.b.c. of fowl 166 when 10 fowls were tested. However, when a number of pig sera were titrated with strain C1, using r.b.c. of this fowl, the N.S.I. titres were still high.

McCrea (1946) reported that the critical temperature for elimination of N.S.I. in rabbit sera lay between 60° C. to 64° C. for 30 minutes. Therefore serum of a normal pig was heated at 60° C. to 65° C. for 20 minutes. This neither eliminated nor reduced the titre of N.S.I. against strain C1.

Virus antigens grown for different lengths of time in the chick embryo were compared to see whether this had any influence on the N.S.I. Serum of a normal pig was titrated using virus (C1) grown for 24 hours or 48 hours. The N.S.I. titres with both the virus preparations were equally high.

No appreciable difference in N.S.I. or antibody titres was found when 1 per cent. sodium citrate was used as diluent instead of normal saline.

Pre-inoculation and post-inoculation sera from Pig A5 were titrated in duplicate. One titration was carried out in the usual way, adding the fowl r.b.c. immediately after the virus, whereas with the other titration, the serum dilutions and virus were incubated at 37° C. for $1\frac{1}{4}$ hours before the fowl r.b.c. were added. With the incubation of serum dilutions and virus, N.S.I. was not reduced.

The sera of Pig A5 were titrated in duplicate. R.b.c. in one titration were allowed to settle at room temperature, and in the other at refrigeration temperature. The antibody titre at refrigeration temperature was twice as much as that at room temperature, while the N.S.I. titre was more than three times greater at refrigeration temperature.

Anderson, Burnet and Stone (1946) showed that the N.S.I. titre of any one normal serum varied considerably according to the strain of virus used. Therefore an experiment was conducted to see whether strains PR8, Barratt or 15 could be used with advantage for titrating pig sera. Titrations were carried out using pre-inoculation and post-inoculation sera of pigs A4 and A5 and allantoic fluids of these strains at the usual concentration, namely, 4 h.d. These results showed that using Barratt there was consider-

able N.S.I. with the pre-inoculation sera, while PR 8 and S 15 were not suitable for detecting antibody against strain C1.

Anderson (1947) reported that N.S.I. was more marked with recently isolated strains than with laboratory strains that were well adapted to growth in the chick embryo. Strain C1 was used after from 4 to 30 passages in the allantoic cavity, but with all passages about the same titres of N.S.I. were encountered.

Treatment of sera with kaolin.

N.S.I. in normal mouse sera was successfully overcome with kaolin, using a technique devised by Chu (1949, personal communication).

A preliminary experiment showed that N.S.I. in normal pig serum could be reduced 1/8 to 1/16 by kaolin treatment, although there was still complete inhibition in a 1 in 10 dilution of all the sera tested. Kaolin treatment also absorbed the agglutinins against normal fowl r.b.c. from the same pig serum.

The effect of kaolin treatment on swine influenza antibody was next investigated. Each pig serum, in volumes of 0·2 ml., was diluted with Sorensen's phosphate buffer *p*H 7·0 to give a 1 in 10 dilution of serum in buffer solution. The diluted serum was added to a centrifuge tube containing 0·2 g. of kaolin powder. The mixture was shaken thoroughly and kept at room temperature (19° C. to 20° C.) for thirty minutes, lightly centrifuged and the supernatant fluid titrated.

Pre-inoculation and post-inoculation sera of pig A4 were treated with kaolin and then titrated. Untreated sera from the same pig were simultaneously titrated. The results given in Table 2 show that the ratio of $\frac{\text{Antibody}}{\text{N.S.I.}}$ with $\frac{\text{Anti-swine influenza}}{\text{normal}}$ sera after kaolin treatment is 20, that is double the ratio between the same sera without kaolin treatment. In an earlier experiment, the ratio with kaolin treated sera of pig A5 was 27, but untreated sera were not titrated.

Table II

Effect of Kaolin Treatment on H.I. Titres

Pig sera	*Kaolin treated*	*Untreated*
Pre-inoculation—A4 ...	20	160
Post-inoculation—A4 ...	400	1600
$\frac{\text{Antibody}}{\text{N.S.I.}}$ ratio	$\frac{400}{20} = 20$	$\frac{1600}{160} = 10$

Use of guinea pig r.b.c.

Although it has been shown that r.b.c. from many mammals are

capable of being agglutinated by influenza viruses (Clark and Nagler, 1943; Chu, 1948), only r.b.c. from the fowl have been used as a routine for H.I. technique by other workers.

In the present study, guinea pig r.b.c. have been used to overcome the N.S.I. encountered in normal pig sera. The use of guinea pig r.b.c. for ferret sera will be discussed in a separate experiment. Serial dilutions of pre-inoculation and post-inoculation pig sera A5 were made in 2 tiles. A dilution of virus containing 4 h.d. against guinea pig r.b.c. was added to one tile, and a dilution containing 4 h.d. against r.b.c. from fowl 166 to the other tile: 2 per cent. guinea pig r.b.c. were then added to the 1st tile, and 2 per cent. r.b.c. from fowl 166 to the 2nd tile. Re-agents in both the tiles were allowed to settle at room temperature. The readings were taken after $1\frac{1}{4}$ hours. The results are shown in Table 3.

TABLE III

H.I. TITRES WITH GUINEA PIG AND FOWL R.B.C.

Pig sera	*g.pig r.b.c.*	*fowl r.b.c.*
Pre-inoculation—A5 ...	<10	60
Post-inoculation—A5 ...	480	640
$\frac{\text{Antibody}}{\text{N.S.I.}}$ ratio	$\frac{480}{<10} = >48$	$\frac{640}{60} = <11$

By using guinea pig r.b.c., N.S.I. encountered with normal pig serum was reduced to less than 10, while a titre of 60 was observed with the same serum titrated with fowl r.b.c.—over six times more. In this experiment, N.S.I. with the pig serum, using fowl r.b.c. was lower than when titrated 3 months before, possibly due to storage of the serum. The antibody titre was slightly lower with guinea pig r.b.c. (480) than with fowl r.b.c. (640). With these particular sera, the ratio of antibody to N.S.I. was, however, more than 48 times with guinea pig r.b.c., as against a ratio of less than 11 with fowl r.b.c.

Guinea pig r.b.c. have been successfully used in the titration of pig sera, their use having overcome the N.S.I. encountered with normal pig sera against swine influenza strain C1. The above observations have been substantiated by titrating a very large number of sera in further experiments in which the antibody levels in pigs were followed after infection or vaccination. It has been of special value in measuring low levels of antibody. These results are reported in further papers of this series.

Comparison of different methods of titration of pig sera.

Six post-inoculation sera from pig A2 to A7 were each treated with kaolin by the technique described. The sera were then

titrated, using r.b.c. from fowl 166. The same sera, not treated with kaolin, were simultaneously titrated, using guinea pig r.b.c. The results are shown in Table 4.

Table IV

H.I. Titres of Swine Influenza Antisera by Two Techniques

Post-inoculation pig sera	*Untreated Sera*	*Sera treated with kaolin*
	g.pig r.b.c.	*fowl r.b.c.*
A2	320	320
A3	160	160
A4	400	320
A5	480	320
A6	120	160
A7	400	200

In 3 out of 6 untreated sera, guinea pig r.b.c. have shown higher titres than kaolin treated sera and fowl r.b.c. With 2 sera the titres were equal, while the 6th showed lower titres with guinea pig r.b.c.

The results obtained with other methods are summarised in Table 5. It must be emphasised that the figures are compiled from experiments performed on different days under different experimental conditions and therefore the titres are not strictly comparable. The Table is included to show the titres obtained with some of the techniques used to overcome N.S.I. encountered with pig sera. In each case, however, pre- and post-inoculation sera were titrated under the same experimental conditions and with the same re-agents. Comparing the ratio $\frac{\text{Antibody}}{\text{N.S.I.}}$, the highest figures have been obtained by the use of guinea pig r.b.c. and the next highest by the kaolin treated sera titrated with fowl r.b.c.

Avoidance of non-specific inhibition in normal ferret sera

The pre- and post-inoculation ferret sera were treated with kaolin in the same way as pig sera. Using fowl r.b.c., these treated sera were titrated with strain C1. Simultaneously, untreated sera were also titrated. The results which are given in Table 6 show that the N.S.I. titre with untreated pre-inoculation serum was as

TABLE V.

COMPARISON OF DIFFERENT METHODS OF TITRATION OF PIG SERA.

Methods of titration.	*Sera from Pig A5*		*Antibody / N.S.I. ratio*
	Pre-Inoculation	*Post-Inoculation*	
(1) Serum dilutions were made in 1 per cent. sodium citrate. (Fowl r.b.c.)	120	960	$\frac{960}{120} = 8$
(2) Serum dilutions and virus incubated at 37°C for 1¼ hours, prior to addition of fowl r.b.c.	300	>2560	$\frac{>2560}{300} = >8{\cdot}5$
(3) Cells allowed to settle at refrigeration temperatures. (Fowl r.b.c.)	400	2560	$\frac{2560}{400} = 6{\cdot}4$
(4) Sera treated with kaolin prior to titration with fowl r.b.c.	15	320	$\frac{320}{15} = 21{\cdot}33$
(5) Cells allowed to settle at room temperature. (Fowl r.b.c.)	60	640	$\frac{640}{60} = 10{\cdot}66$
(6) Cells allowed to settle at room temperature. (G. pig r.b.c.)	<10	480	$\frac{480}{<10} = >48$

Titres are expressed as reciprocal of serum dilution showing one + haemagglutination.

TABLE VI

H.I. TITRES OF FERRET SERA WITH DIFFERENT TECHNIQUES.

Ferret sera before and after inoculation	*Fowl r.b.c.*		*G. pig r.b.c.*	
	Untreated	*Kaolin treated*	*Untreated*	*Kaolin treated*
Before	800	100	80	<10
After	12800	8500	15360	15360
Antibody / N.S.I. ratio ...	$\frac{12800}{800} = 16$	$\frac{8500}{100} = 85$	$\frac{15360}{80} = 192$	$\frac{15360}{<10} = >1536$

high as 1/800; with kaolin treatment, this was reduced to 1/100. The ratio $\frac{\text{Antibody}}{\text{N.S.I.}}$ with kaolin treated sera was 85 as against 16 with untreated sera. The treatment with kaolin only slightly reduced the titre of antibody in the post-inoculation serum.

Untreated and kaolin treated ferret sera were next titrated using guinea pig r.b.c. The N.S.I. titre of 1/80, observed with untreated pre-inoculation serum, was reduced to less than 1/10 by kaolin treatment of the serum. Kaolin treatment did not reduce the antibody titre in this experiment. The ratio $\frac{\text{Antibody}}{\text{N.S.I.}}$ with kaolin treated sera was at least 8 times greater than it was using untreated sera (see Table 6).

Summing up the results of these experiments, titration of ferret sera with guinea pig r.b.c. after kaolin treatment, was found to be the most satisfactory technique to overcome N.S.I. without appreciably affecting the antibody titre. This technique was employed for the titration of all ferret sera during the course of further investigations on respiratory diseases of pigs.

DISCUSSION

Haemagglutination and haemagglutination-inhibition techniques have been used extensively in the field of virus research, especially with the influenza and Newcastle disease group of viruses. Standard techniques and various modifications of them, have been used for virus and antibody titration to suit various requirements. Non-specific inhibition (N.S.I.) in normal sera of man and animals, however, limits the use of the haemagglutination-inhibition technique, and the results are rather difficult to interpret when both N.S.I. and antibodies, especially of unknown or doubtful history, are present in a serum.

Tests were done with various methods which other workers found useful in minimising N.S.I., but none was satisfactory with the sera and the virus strains used in this investigation.

Most other workers have used only fowl r.b.c. for haemagglutination-inhibition tests. Although viruses of the influenza group are known to agglutinate mammalian r.b.c., the latter have not been used for routine tests. In the present studies, N.S.I. in pig sera was eliminated by the use of guinea pig r.b.c. With ferret sera, the use of guinea pig r.b.c. reduced the N.S.I. greatly, but prior treatment with kaolin was necessary to eliminate it.

In testing sera from man and other animals, it would be interesting to see if guinea pig and other mammalian r.b.c. could be used satisfactorily with newly isolated influenza strains, or strains with which high N.S.I. is given by normal sera when fowl r.b.c. are used.

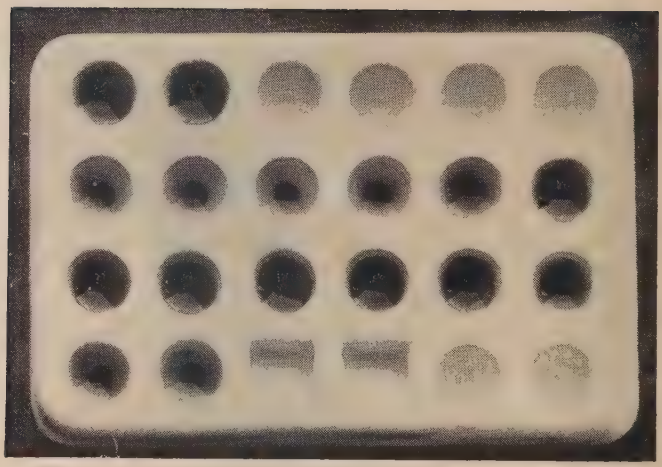

FIG. 1

1/640	1/1280					Serum dilutions, virus and r.b.c.
+++	+++					
1/10	1/20	1/40	1/80	1/160	1/320	
–	–	–	–	±	±	
1/10	1/20	1/40	1/80	1/160	1/320	Virus dilutions and r.b.c.
±	±	±	±	±	±	g. pig r.b.c. control.

SUMMARY

Infected allantoic fluid of chick embryos was superior to infected mouse lung or pig lung as an antigen for virus haemagglutination and haemagglutination-inhibition tests.

Non-specific inhibition was encountered in sera of normal pigs and other animals when they were titrated against strain C1 of swine influenza virus using fowl r.b.c. Neutralisation tests in mice and conglutinating-complement absorption tests showed that this inhibition was not due to specific antibody. The difficulty was overcome by using guinea pig r.b.c. for H.I. tests with pig sera. With this technique the N.S.I. titre was <10 whereas antibody titres were almost the same as when fowl r.b.c. were used.

N.S.I. in ferret sera was eliminated by treating the sera with kaolin and then using guinea pig r.b.c. for the titration.

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STUDIES ON RESPIRATORY DISEASES OF PIGS

II. Antibody response to adjuvant vaccine against Swine Influenza

By

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INTRODUCTION

Shope (1932) reported that the virus of swine influenza when administered intramuscularly in the active state, immunised pigs against swine influenza. In 1936, he found that in field trials with this vaccine, where there were no facilities for keeping the inoculated piglets separate from other susceptible piglets, the latter developed the disease. He concluded that "The use of live swine influenza virus as a prophylactic agent may be less dangerous to the recipient of the virus than it is to other susceptible individuals with which the recipient may come in contact during the course of immunisation." Working in Russia Agapov (1940) also produced immunity in pigs by the subcutaneous inoculation of active swine influenza virus.

McLean, Beard, Taylor, Sharp and Beard (1945 a, b, c, d) inoculated pigs with a highly concentrated and partially purified virus, inactivated either by formalin or ultra-violet light, and also formolised virus absorbed on different amounts of alum. They reported that repeated small doses produced a higher and more lasting antibody response than did a single large dose. The effect varied with the interval between inoculations. Vaccines prepared with formalin or ultra-violet irradiation behaved alike and absorption on alum did not greatly enhance the titre or prolong the antibody level. The antibody response of swine to vaccination with formolised influenza virus suspended in saline solution was characterised by a swift increase in antibody titre and by a subsequent rapid decline in the titre to low levels.

Because of the rather rapid loss of circulating antibody against influenza virus in man after vaccination, various substances have been combined with the inactivated virus for the purpose of prolonging the period of high antibody level. Friedwald (1944 a, b) reported excellent results with influenza A (PR8 strain) when adjuvants were incorporated with formolised allantoic fluid virus using mice, ferrets and rabbits as test animals. Better results were obtained by the use of acid-fast organisms, Falba[1] and paraffin oil mixed with the virus, than with these adjuvants but without acid-

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[1] Falba is said to be a mixture of bees-wax, paraffin oils of various viscosities and certain extractions from lanolin. It is distributed by Pfaltz and Baker Incorporated, New York, who kindly supplied the sample used in this work.

fast organisms. These mixtures caused much local reaction which, however, was less when acid-fast organisms were excluded. Henle, Henle, Hampil, Maris and Stokes (1946) studied the effect of similar adjuvants excluding the acid-fast organisms. Abscesses were formed in 2 out of 80 vaccinated human beings, but the vaccine gave rise to very high antibody titres which were maintained for long periods.

Studies on the transfer of protective substances or antibody from sow to offspring have been reported by various workers. Nelson's studies with vaccinia virus (1932, 1934) have clearly shown that the porcine placenta is impermeable to the transfer of any appreciable amount of protective substances to the foetuses, and that those substances that are passed to the offspring are transferred via the colostrum. These results were confirmed by Young and Underdahl (1949) using live swine influenza virus administered intramuscularly as a vaccine. Further, they showed that the colostrum contained higher titres of antibody than sow's serum, as titrated by haemagglutination inhibition (H.I.) test.

In the work about to be described, vaccines consisting of swine influenza virus concentrated and mixed with Falba and mineral oil gave rise to high levels of antibody. Two vaccinated sows passed antibody to their offspring via the colostrum.

MATERIALS AND METHODS.

Preparation of concentrated formolised vaccines.

A batch of 35 embryonated eggs, 11 days old, was inoculated in the allantoic cavity with 0·1 ml. of a 1 in 30 dilution of allantoic fluid infected with strain C1 of swine influenza virus. They were incubated at 37° C. for 48 hours, and then chilled overnight at 4° C. The allantoic fluids from these eggs were tested for haemagglutination at a 1 in 50 dilution, and for bacterial sterility. Formalin was added to 200 ml. of pooled allantoic fluid to give a final concentration of 1 in 10,000 and the fluid was stored at 4° C. overnight.

It was then centrifuged at 1,000 r.p.m. for 10 minutes and the sediment discarded. The supernatant was centrifuged for 1½ hours at a speed of 11,400 r.p.m. in an angle centrifuge. The resultant supernatant was removed, and the sediment re-suspended in 10 ml. of 0·05 sodium phosphate buffer at *p*H 7·0 (Stanley, 1945). The re-suspended virus was centrifuged at a speed of 2,000 r.p.m. for 7 minutes, the sediment discarded and the supernatant centrifuged again at a speed of 11,400. The supernatant was removed and the sediment re-suspended in 2 ml. of buffer. This suspension was centrifuged at 2,000 r.p.m. for 7 minutes and the sediment discarded. Formalin was added in final concentration of 1 in 1,000 and the mixture incubated at 35° C. for 30 minutes and stored at 4° C. It was tested for infectivity by inoculation into chick embryos undiluted, and at a 1 in 100 dilution and found inactive.

This preparation will be referred to as concentrated formolised vaccine, batch 1.

The haemagglutination titres of the original pool of the 2 supernatants and of the 2 concentrated suspensions with fowl r.b.c. are shown in Table I.

TABLE 1

Pool allantoic fluid	*1st supernatant*	*1st concentrate (X10)*	*2nd supernatant*	*Final concentrate (X100)*
100	15	150	40	800

A second lot of concentrated virus, formolised vaccine batch 2, was prepared in the same manner as the first batch, except that the sediment after the first round of high speed centrifugation was suspended in the same volume of buffer solution as the original pool of allantoic fluids. The vaccine was shown to be non-infective by inoculation into chick embryos as before.

Preparation of Adjuvant vaccines.

Batch 1 was prepared by the technique similar to that described by Friedwald (1944a). The three ingredients, formolised virus. Falba and liquid paraffin, were used in the proportions of 1, 1 and 4 respectively. Falba and liquid paraffin were previously sterilised.

Batch 2 was prepared by the same technique used for preparing batch 1 after the concentrated virus batch 1, had been stored for 10 days. This was then mixed with a small amount left from adjuvant vaccine batch 1. The mixture will be referred to as "adjuvant vaccine batch 2".

Batch 3 was prepared from formolised vaccine batch 2, by the same technique as adjuvant vaccine batch 1, using the same proportions of formolised virus, Falba, and liquid paraffin

Titration of pig sera.

Pigs were bled from the anterior vena cava by the technique of Carle and Dewhirst (1942). All the sera were inactivated at 56° C. for 30 minutes, and stored at 4° C. The sera were titrated by the modified haemagglutination inhibition (H.I.) technique already described (Gulrajani, 1951). Guinea pig r.b.c. instead of the usual fowl r.b.c. were used in the H.I. tests to overcome the non-specific inhibition encountered with Cambridge (C1) strain of swine influenza virus.[1] In the graphs H.I. titres of less than 10 have been plotted as zero.

Preparation of whey.

The colostrum or milk was curdled by adding rennet. After

[1] Isolated by Blakemore and Gledhill (1941).

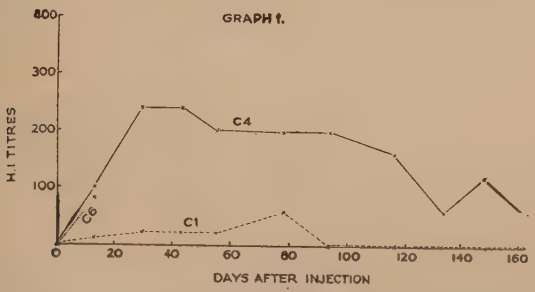
incubation at 45° C. for ten minutes, it was lightly centrifuged, and clear supernatant was used for H.I. titrations.

VACCINATION EXPERIMENTS

Piglet C4 was injected subcutaneously in the left flank with 0·3 ml. of adjuvant vaccine (batch 1), while piglet C6 was injected in each flank with 0·3 ml. of the same adjuvant vaccine divided into two doses of 0·15 ml. After injection, the site was massaged gently to disperse the vaccine so as to avoid forming lumps. Piglets C1 and C2 were injected subcutaneously in the left flank each with 0·05 ml. of concentrated formolised vaccine (batch 1) diluted in saline to give a final volume of 0·3 ml. The piglets were seven days old when injected.

Piglet C9, a litter mate of these piglets, was kept in contact as an unvaccinated control. Rectal temperatures of all five piglets were recorded twice daily for two days before and ten days after vaccination. No appreciable rise in temperature was recorded.

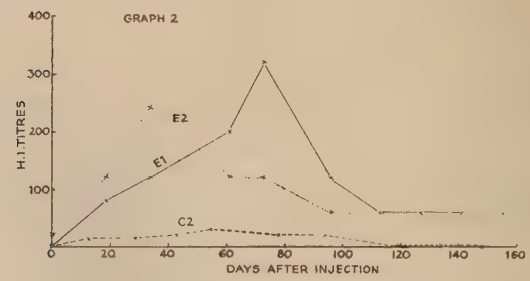
After a week, small hard lumps were observed in both flanks of piglet C6, while no local reaction was visible in the other piglets. Piglet C6 died accidentally when it was bled 13 days after injection. On post mortem examination the lungs were normal. No swellings or any other local reaction were seen at the sites of inoculation. Pre-inoculation sera from all these piglets were obtained on the day they were born: post-inoculation samples were taken at approximately fortnightly intervals. The H.I. titres of piglets C1, C4 and



GRAPH 1. H.I. titres after injection with formolised vaccine batch 1 (pig C1) and adjuvant vaccine batch 1 (pigs C4 and C6).

C6 are shown in graph 1. H.I. antibody was not present in the serum of control piglet C9 taken 13 days after the other piglets were injected.

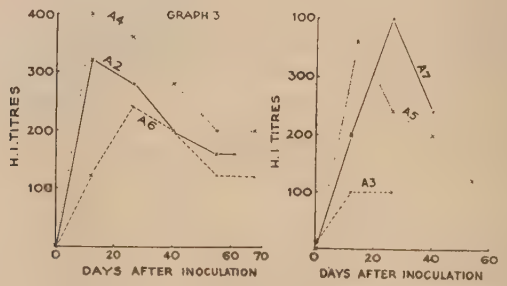
Piglets E1 and E2 (one week old), which were unrelated to the "C" piglets, were injected subcutaneously in the flank each with 0·3 ml. of adjuvant vaccine (batch 2) after it had been stored for 12 days at 4° C. The extent and persistence of H.I. titres in the sera of piglets E1, E2 and C2 are shown in graph 2. For convenience of



GRAPH 2. H.I. titres after injection with formolised vaccine batch 1 (pig C2) and adjuvant vaccine batch 2 (pigs E1 and E2).

comparison, the H.I. titres of pigs A2 to A7 after experimental infection with virus of swine influenza are shown in graph 3.

After injection with adjuvant vaccine (batch 1), antibody was present in the samples of serum taken from piglets C4 and C6 on the 13th day. In piglet C4, the titre reached to 240 on the 29th day and was maintained at this level to the 43rd day, but fell to



GRAPH 3. H.I. titres after experimental infection with virus of swine influenza. Pigs A2—A7.

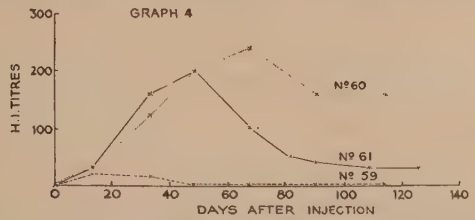
200 on the 55th day. The titre remained at 200 to the 94th day, and fell to 160 on the 117th day; thereafter, it fluctuated up to the 162nd day, as shown in graph 1. Three more serum samples taken on the 176th, 196th and 212th days after inoculation again showed titres of 160 but these are not shown in the graph.

The peak of titre in piglet E1 was reached on the 73rd day, while in piglet E2, it was reached on the 34th day. A titre of 60 was maintained for 155 days, that is, for 22 weeks after the injection.

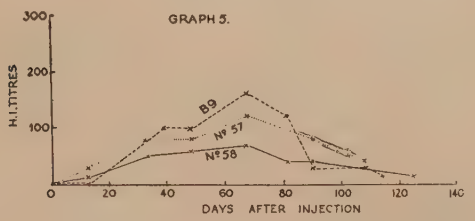
The H.I. titres following inoculation with formolised vaccine have been very poor as compared with those obtained with adjuvant vaccine. There has been only one reading of 60 in piglet C1 on the 78th day.

The H.I. titres which accompanied the clinical disease produced experimentally with the virus of swine influenza in piglets A2 to A7, were much higher for 40 days, but on the 55th day, they dropped level with the titres produced in piglets C4, E1 and E2 injected with adjuvant vaccines. Titres produced after a subclinical infection with virus of swine influenza were lower than those produced after adjuvant vaccines (see pig D1, graph 9).

A group of five one-week-old piglets (viz. nos. 57, 58, 60, 61 and B9) from three different litters were each injected subcutaneously in the flank with 0·3 ml. of adjuvant vaccine (batch 3). A slight swelling in piglet B9 was observed on the 4th day after injection, but could not be detected on the 13th day. Piglet 59 was vaccinated with 0·05 ml. of concentrated formolised virus (batch 2) diluted in saline to give a total volume of 0·3 ml. The site and route of injection used for this piglet were the same as for the other 5 piglets. A sample of serum was obtained from each piglet before and at approximately fortnightly intervals after inoculation. The H.I. titres of sera from these piglets are shown in graphs 4 and 5.



GRAPH 4. H.I. titres after injection with formolised vaccine batch 2 (pig 59) and adjuvant vaccine batch 3 (pigs 60 and 61).



GRAPH 5. H.I. titres after injection with adjuvant vaccine batch 3.

With this batch of vaccine also, better serological results were obtained by the use of adjuvant vaccine than with formalised vaccine, the dose of virus being the same in both vaccines. Four out of five piglets in the adjuvant group showed definite evidence of H.I. antibody on the 13th day post inoculation, while antibody was not present in the serum of piglet B9 obtained at the same time. The H.I. titres subsequently increased. The peak in piglet 61 was reached at 48 days, and in the remaining four piglets, 67 days after inoculation. The titres had definitely fallen by the 81st

day, or thereafter. These figures also showed marked individual variation. For example, serum obtained from piglets 60 and B9 114 days after inoculation showed a very wide difference; in piglet 60 the titre was 160, whereas in piglet B9 it was 15.

The H.I. titres produced with formolised vaccine (batch 2) have also been extremely poor, though it is possible that the peak of antibody production following the vaccine has been missed. The experience of McLean, et al. (1945 a) is, that following the injection of formolised virus vaccine, the peak was reached between the 7th and 9th day. In my experiments, the formolised vaccines were, however, used only to compare the titres with those obtained with adjuvant vaccines.

Sow A was injected into the subcutaneous fascia with 0·3 ml. of adjuvant vaccine (batch 2). Rectal temperatures were recorded twice daily for two days before and ten days after injection, but no appreciable rise was observed. The vaccination caused some local reaction on the 5th day. The part around the site of injection was painful and hot, and a very small lump was formed. On the 7th day the sow showed slight lameness, which lasted three days: however, two weeks after injection and thereafter, there was no pain or tenderness, and no further reaction was observed.

Sow A was injected under the impression that she was pregnant. After 40 days, she was found not to be pregnant and was served again. She farrowed 152 days after injection, giving birth to a litter of ten piglets (nos. G1 to G10). A pre-suckling serum sample was obtained from 4 piglets, viz. G1, G2, G3, G4. During the night, she lay on piglet G4 and killed him. A sample of serum was collected from 8 out of 9 piglets 2 days after birth, but a sample could not be obtained from piglet G3 at this stage. The 9 piglets were all bled when they were 9 days and 28 days old. The H.I. titres of these sera, except piglet G8 whose serum was not titrated at this stage, are set out in Table 2 which shows that the H.I. antibody in the piglets from a vaccinated sow is transferred through the colostrum only. A high titre of antibody was passed to piglets, as seen in serum samples obtained 2 days after birth. On the 9th day, H.I. titres had decreased in some piglets, while the titres were the same in others. On the 28th day, however, they had definitely decreased in all the piglets. Sow A farrowed again 325 days after inoculation with adjuvant vaccine. It was demonstrated that antibody was transferred to the 2nd litter also. A titration of samples of sera taken from 2 piglets (L1 and L2) of this litter 38 days after birth showed that both had H.I. titres of 60.

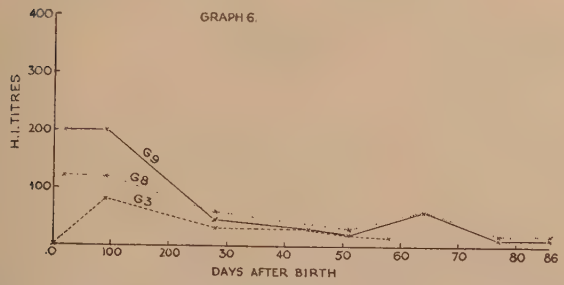
The piglets of the first litter were arranged in three groups, viz., group I (G3, G8 and G9), group II (G2, G6 and G10) and group III (G1, G5 and G7). Group I was left as unvaccinated controls. Group II was injected with adjuvant vaccine (batch 3), which had been stored for 40 days at 4° C., and Group III with adjuvant vaccine (batch 2), which had been stored for 186 days at

TABLE II

H.I. TITRES OF PIGLETS FROM VACCINATED SOW.

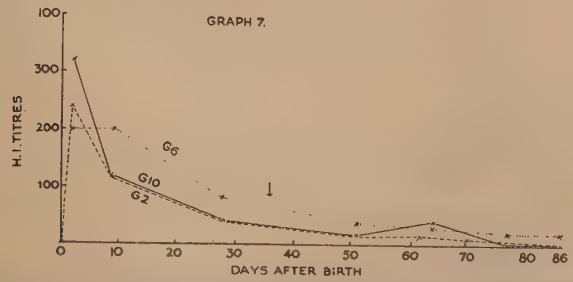
Piglet No.	Days after birth.			
	0	2	9	28
G1	<10	400	320	80
G2	<10	240	160	80
G3	<10		160	60
G4	<10			
G5		320	320	120
G6		320	320	160
G7		320	240	80
G9		>320	320	120
G10		>320	160	80

0 indicates serum samples taken before sucking.



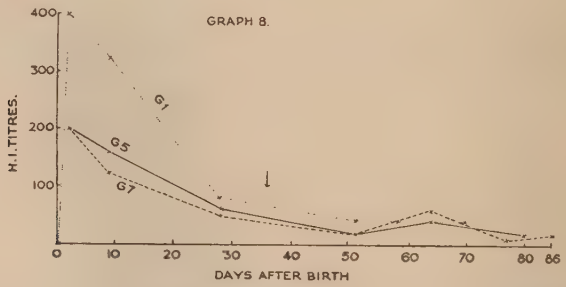
GRAPH 6. H.I. titres of sera from pigs born of Sow A which had been injected with adjuvant vaccine. Pigs in this group (I) were not themselves vaccinated.

4° C. The piglets in group II and III were vaccinated subcutaneously in the flank each with 0·3 ml., when they were 36 days old. Samples of serum have been obtained from piglets comprising the three groups at approximately fortnightly intervals. H.I. titres of the three groups of piglets are shown in graphs 6, 7 and 8.



GRAPH 7. H.I. titres of sera from pigs born of Sow A which had been injected with adjuvant vaccine. Pigs in this group (II) were injected with adjuvant vaccine batch 3 on the day indicated by arrow.

These titrations were done at the end of the experiment and all the sera from each piglet were titrated at the same time. Comparing the results shown in graphs (6, 7 and 8) and Table II, it will be seen that in most cases titres of sera obtained on the 2nd, 9th and 28th days after birth have decreased to some extent, possibly due to storage.



GRAPH 8. H.I. titres of sera from pigs born of Sow A which had been injected with adjuvant vaccine. Pigs in this group (III) were injected with adjuvant vaccine batch 2 on the day indicated by arrow.

After vaccination, there was no significant difference in H.I. titres of piglets of unvaccinated and vaccinated groups. The adjuvant vaccines were unfortunately not inoculated at the same time in normal piglets to check that they had maintained their potency during storage. The only available evidence on the keeping qualities of these vaccines is that adjuvant vaccine (batch 2) after storing for 12 days at 4° C. gave satisfactory results. It is well known, however, that formolised influenza vaccines keep for many months. The other possible explanation for the failure to obtain an antibody rise is that the antigens in the adjuvant vaccines, which are absorbed slowly, might have been neutralised by the antibody present in the sera of these piglets. It is not possible to say what is the correct interpretation of that part of the experiment.

Samples of colostrum or milk were taken from sow A on the day of farrowing and on the 1st, 2nd, 4th, 6th, 8th, 13th and 20th day after. The whey was removed from these samples by the technique described earlier. After heating at 56° C. for 30 minutes, the whey was titrated by the modified H.I. technique. The titres are shown in Table 3.

TABLE III

H.I. TITRES OF WHEY OF VACCINATED SOW.

	Days after farrowing.							
	0	*1*	*2*	*4*	*6*	*8*	*13*	*20*
Sow A ...	1280	240	240	120	60	40	40	40

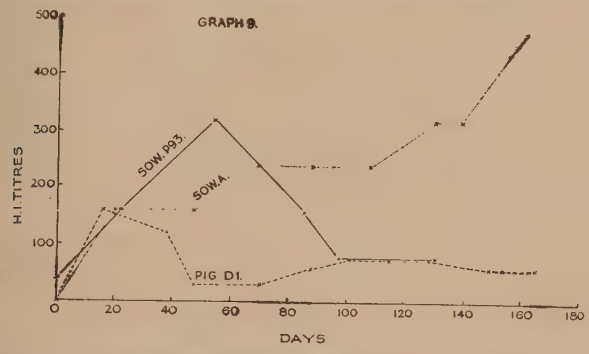
TABLE IV

H.I. TITRES AFTER INOCULATION WITH ADJUVANT VACCINE

	Days after inoculation										
	0	20	47	69	88	108	129	139	161	187	203
Sow A	<10	160	160	240	240	240	320	320	480	320	320

The H.I. titres of serum samples taken from sow A are shown in Table 4 and graph 9. In graph 9, H.I. titres of pig D1 (mentioned previously) and of sow P93 are also included.

Sow P93 was injected with adjuvant vaccine (batch 2). She farrowed 20 days after vaccination and passed antibody to her piglets. Serum taken on the 9th day from 2 piglets of this sow



GRAPH 9. H.I. titres after injection with adjuvant vaccine batch 2 (sows A and P93) and after subclinical infection with virus of swine influenza (pig D1).

neutralised a 1 in 100 suspension of infective mouse lungs, while serum of a normal piglet failed to neutralise the same amount of the virus. The mouse neutralisation technique used was described earlier. (Gulrajani, 1951.)

DISCUSSION

The virus vaccines which have been used hitherto can be divided into three broad groups as follows. (1) Vaccines containing live viruses which may or may not be attenuated. Although very satisfactory immunological and serological results have been reported with such vaccines, there are often dangers involved in their use. (2) Inactivated virus vaccines which are quite safe, but often give poor immunological and serological responses. (3) Inactivated viruses mixed with adjuvants which are safe to administer and have given very satisfactory serological results. The main objection against these vaccines is the occurrence of local reaction.

The adjuvant vaccine prepared with formolised swine influenza virus and used in the above experiments is safe to administer, has given very satisfactory serological results and has caused no serious local reaction. The results obtained are encouraging and suggest a field for further investigation. If these results be confirmed in work with other diseases, the use of adjuvants in virus vaccines in animals may have a wider application. Too much stress cannot, however, be laid on my results with swine influenza, especially since no immunity tests have been carried out.

SUMMARY

Week-old piglets were vaccinated, some with formolised virus and some with a similar vaccine to which adjuvants had been added. The H.I. antibody response to the adjuvant vaccine was much the better, and in some cases equalled the response to infection with live virus. Immunity tests on piglets thus vaccinated have not, however, been carried out. Two sows were vaccinated with adjuvant vaccine, titrations carried out on the haemagglutination inhibition (H.I.) antibody in their sera and, in one sow, on the colostrum. The antibody of the colostrum, which was much higher in titre than that of the serum, has been transferred to piglets. The results of H.I. tests were confirmed by showing that the sera of two of the piglets neutralised virus in neutralisation tests carried out in mice.

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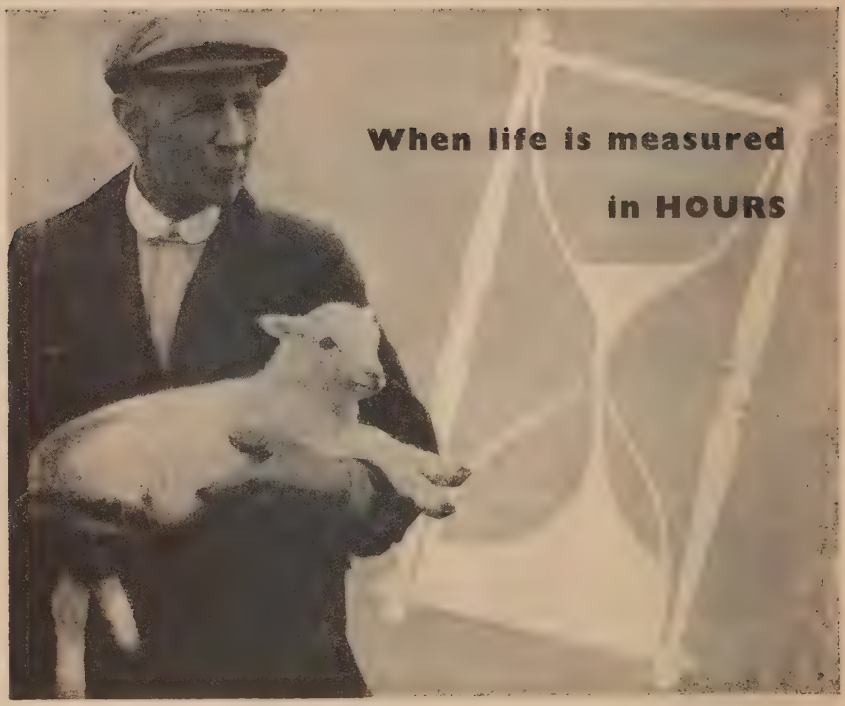

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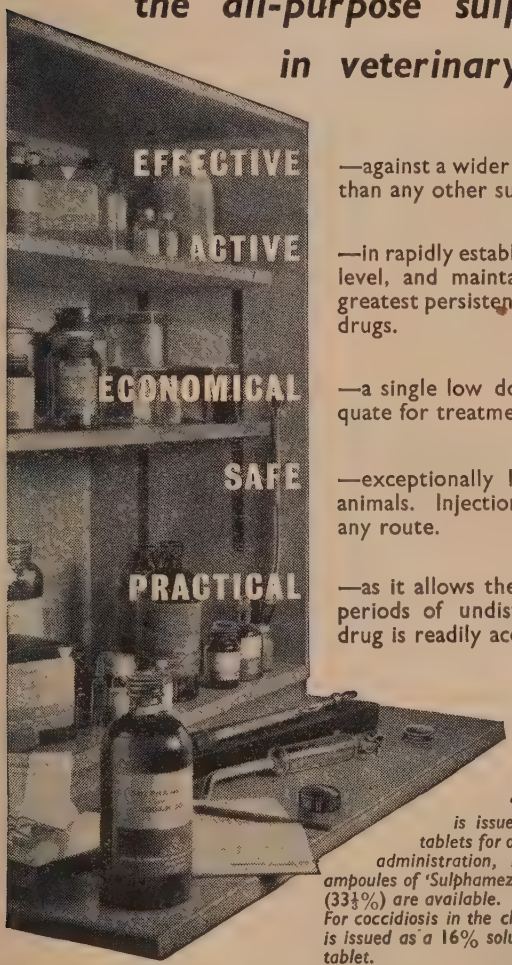

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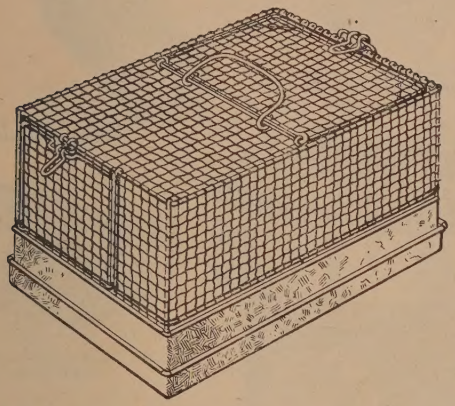

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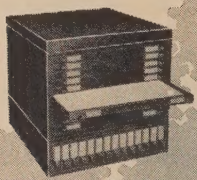

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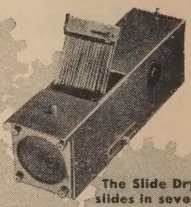

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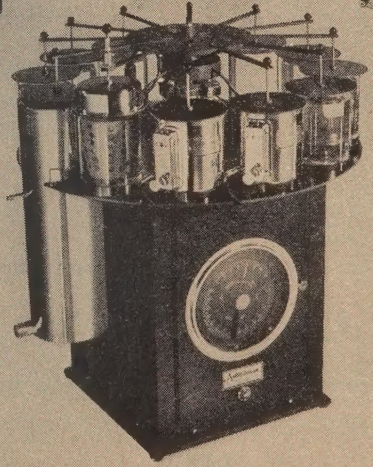

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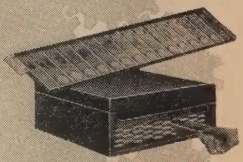

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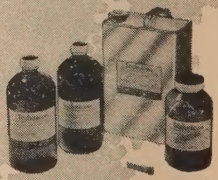

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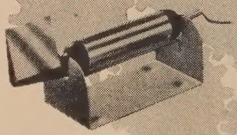

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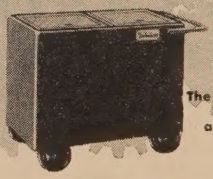

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